A LABORATORY MANUAL OF PHYSIOLOGICAL CHEMISTRY

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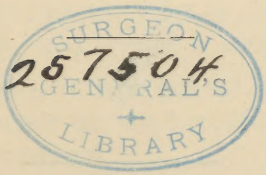
OF

PHYSIOLOGICAL CHEMISTRY

Prepared for the use of the Students of Northwestern Medical School

BY

CHESTER J. FARMER
Professor of Chemistry
Northwestern Medical School
With the cooperation of Staff Members



Student's Name.....

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METHOD OF WEIGHING

1.—Determine the zero-point of the balance by setting it in motion without any load on the pans. Observe and write down the turning points, or extreme positions, of the pointer on the scale, of an uneven number of swings, say five or seven. These readings must be taken with the balance case closed. Disregard the first two readings as they are inaccurate. In order to give the same algebraic sign to all of the observed readings, it is best to number the divisions on the scale from left to right: 0 to 20. In other words, call the long line in the center 10 (the one over which the pointer usually stands), then 9, 8, 7, etc.; 2, 1, 0 to the left and the 10 ascending numbers, 11, 12, 19, 20, to the right. Average the left swings and the right swings separately, then get a mean by averaging the two average values. Take this as the zero point without load (see example). Assume it is 10.99.

2.—Now determine the sensitiveness of the balance for the object to be weighed. That is, you are about to determine how many scale divisions the pointer traverses or is displaced by changing the weight 1 milligram. This enables you to calculate the fourth decimal place of your weight. To do this, place a clean crucible on the left pan, add weights to the right pan until equilibrium is established as nearly as possible when using only the milligram division on the beam or milligram weights in the box, as the smallest denomination. Suppose with this first point of rest a mean scale reading of 12.35 with a weight of 22.052 grams is obtained. Since the weight is a little light, add 1 milligram on the beam with the rider or from the box, making the weight 22.053 gms., and assume a mean scale position of 9.71.

The sensitiveness is therefore $12.34 - 9.71 = 2.64$ scale divisions. In case you add too much weight at first, the process will, of course, be just reversed.

As the zero-point of the balance was at 10.99 and the point of rest with a load of 22.052 grams was 12.35, it follows that the object was heavier than the weights in the scale pan. In fact, the excess of weight of the object was sufficient to move the pointer $12.35 - 10.99 = 1.36$ scale divisions.

The above data enables us to calculate the true weight of the object to the fourth decimal place, as follows: 2.64 scale divisions correspond to 1 mg., then 1.36 scale divisions correspond to that fraction of a milligram which must be added to 22.052 gms., to obtain the true weight of the crucible in air. (If the first weight had been heavier, the reasoning is the same, except that the fractional part of the milligram should be subtracted.) That is:

$$1 : 36 : 2.64 :: x : 1$$
$$x = 0.5 \text{ mg.}$$

Therefore the true weight of the crucible in air is 22.052 plus 0.0005, which is 22.0525 gms.

The students' record of weighing for the above case should be kept in the following manner:

	Zero Point		1st Point of Rest 22.052 gms.		2nd Point of Rest 22.053 gms.	
	Left	*Right*	*Left*	*Right*	*Left*	*Right*
	4.2	17.6	5.8	18.7	3.5	15.8
	4.6	17.1	6.2	18.3	3.8	15.4
	5.1	....	6.6	....	4.2	....
Sum	13.9	34.7	18.6	37.0	11.5	31.2
Mean	4.63	17.35	6.2	18.5	3.83	15.6

Sum of means..	21.98	24.7	9.71
Means average .	10.99	12.35	19.43

Sensitiveness = 12.35 − 9.71 = 2.64 scale divisions.

Weight deflection from zero point = 12.35 − 10.99 = 1.36 div.

Fractional weight = $\frac{1.36}{2.64}$ = 0.54 mgs.

Weight of crucible in air = 22.052 plus 0.0005 = 22.0525 gms.

THE USE OF ANALYTICAL WEIGHTS

The student is to ask for the box of weights at the store room window, giving the number of his balance. He must sign a loan slip, and is charged with the set until returned. The weights will be inspected immediately and, if found complete and undamaged, the slip will be returned. The weights must always be handled with forceps provided in the box, never with the fingers or crucible tongs.

The large weights range from 1 to 50 grams, while the small flat weights covered by a glass plate are fractional parts of the gram. The fractional weights are marked either in fractions of a gram or as multiples of a milligram, thus a weight marked 0.1 and 100 are the same, the first system calls it one-tenth of a gram or a decigram, while the latter system indicates the fact that it is also one hundred milligrams.

Again a fractional weight may be marked 0.01 (one centigram) or 10 (ten milligrams).

The best method of obtaining weights less than 10 milligrams is by the use of the bent wire called a "rider," which slides along the right arm of the balance beam. The graduations vary, but in all cases the figures indicate milligrams. A little study will enable the student to properly evaluate the smaller lines between milligram markings on the beam. A few balances have only six milligram divisions on the beam. They require the use of a five-milligram weight (placed on the weight pan) in conjunction with the rider to obtain values between 5 and 10 milligrams.

For the sake of accuracy, learn to count up your weights from the empty box, at first writing down each one removed in the note book. For example, suppose you remove the following weights: 10-gm., 5-gm., 2-gm., 1-gm., 0.1-gm., 0.05-gm., and have moved the rider to a point indicated on the beam by figure 7. Record weights as above, check from the empty box to see if correct, then as you remove them from the scale pan, draw a pencil mark through the weight recorded. By so doing, you will often find a mistake before a determination has been spoiled, or much subsequent work depending upon this one weight lost, as in weighing out material for a standard solution.

The weight obtained by using the above individual weights should be recorded as 18.3570 grams. The fourth decimal place is obtained as directed above under the method of "swings."

ACIDIMETRY AND ALKALIMETRY

3.—Preparation of N/2 Oxalic Acid Solution. Grind in a clean mortar about 10 grams of purest obtainable oxalic acid crystals. Spread on a 4-inch watch crystal and expose to the air for 15 minutes. At the end of this time transfer to a wide-mouth, glass-stoppered bottle. This treatment is to assure the

acid of a definite amount of water of crystallization. It should correspond to the formula $C_2H_2O_4$-$2H_2O$.

From this material prepare 250 c. c. of N/2 oxalic acid, as follows: Carefully clean and dry a small beaker of 150 cc. capacity. Place it on the left pan of the balance and accurately obtain its weight to the fourth decimal. The amount of oxalic acid required for the solution is 7.8780 gm. Why this weight? Having obtained the weight of the beaker, add to it the required weight for the oxalic acid, and reset the weights on the balance to this figure.

Carefully add to the beaker sufficient oxalic acid to *exactly counterbalance this weight.* (Accurate to the fourth decimal.) Record your weighings. Be careful not to spill oxalic acid on the pan or in the balance case. Remove the beaker from the pan, add 50 cc. of distilled water and gently warm, using a wire gauze, over a very low flame.

Clean the 250 cc. volumetric flask with cleaning fluid, wash out thoroughly with tap water, finally rinse twice with distilled water. Have a funnel which will fit conveniently into the neck of the volumetric flask.

Now transfer, using a stirring rod, and *without the loss of* a drop, the solution in the beaker to the flask. Add more distilled water, warm as before, transfer to the flask. Repeat until all of the material has been brought into solution and transferred to the flask. Finally rinse the beaker very thoroughly into the flask, using small portions of distilled water and repeating the washing 4 to 5 times. The funnel is rinsed and removed. Caution: Watch the volume of fluid in the flask. It must now be at least 1 inch below the graduation on the neck.

The flask has a capacity of 250 cc. only at a given temperature. This is indicated just below the figure showing its capacity. To bring the flask to this temperature, insert a clean thermometer, then immerse the bowl of the flask in water using ice if necessary, or warm water as required. The flask should be gently rotated and its contents brought to the desired temperature.

Now remove the thermometer from the volumetric flask,

directing a small stream of water from the wash bottle on to it as it is being withdrawn, so that the rinsings return to the flask. Remove when rinsed. *Caution*—Do not fill above graduation on neck!

The remaining volume of water is added from the wash bottle, or by means of a pipette until the meniscus rests exactly on the calibration mark. Any droplets in the neck above this point are removed by a roll of filter paper. The flask now should be stoppered, and inverted occasionally until diffusion is complete. At a later time after the solution has thoroughly diffused, it must be transferred to clean dry bottle. The label should show the name, concentration, and date on which the solution was prepared.

4.—Preparation of an N/2 Sodium Hydroxide Solution

The strong sodium hydroxide solution in the laboratory contains about 40% NaOH. From it prepare about 1200 cc. of a solution so diluted as to be a little stronger than half normal. Stopper and mix thoroughly. Allow the solution to stand at least three hours with occasional shaking.

Determine the strength of the solution so prepared by titrating it several times against the standard N/2 oxalic acid. To titrate this solution, pipette out 25 cc. of standard oxalic acid solution into a 200 cc. beaker. Add 25 cc. distilled water and one drop of phenolphthalein as an indicator. Carefully rinse a clean rubber-tipped burette with small portions of the alkali, and then fill with the same solution. Care should be taken to expel any air from the rubber tip of the burette before the alkali is drawn down to the graduated scale. Read the burette and record the volume. Carefully allow the alkali to flow into the oxalic acid contained in the beaker, constantly stirring with a glass rod. The alkali should be added slowly at first, and later a very few drops at a time. The first faint pink color permanent for 15 seconds should be taken as the end point. Read the burette and subtract the first reading. This gives the number of cc. of the alkali solution which are equivalent to 25 cc. of an exactly N/2 oxalic acid solution. Repeat the titration until checks are obtained, then from the results of the determinations, calculate how much of this solution must be used for the preparation of 1000 cc. of an exactly half normal sodium hydroxide

solution. (Consult the instructor.) Prepare this by suitable dilution of the standardized sodium hydroxide solution, and after thorough diffusion has taken place, check the correctness of your solution against the standard oxalic acid solution. If correct, transfer the liquid to a clean, dry bottle. Stopper and preserve in a properly labeled bottle.

5.—Exercise

By means of the N/2 NaOH solution, determine the concentration of the unknown HCl solutions furnished, using alizarin red as an indicator. Report the result to the instructor before proceeding with the next experiment.

6.—Preparation of N/2 Hydrochloric Acid

The concentrated hydrochloric acid of the laboratory contains about 35% HCl. First make a rough dilution of the required amount to 1200 cc. From this prepare 1000 cc. of an N/2 HCl in the same manner as the N/2 NaOH solution was prepared from the concentrated sodium hydroxide, using the N/2 NaOH solution as a standard and alizarin red as indicator. If correct, transfer the N/2 HCl solution to a clean, dry bottle. Label and preserve.

7.—Preparation of N/10 Solutions

Prepare 1000 cc. of N/10 HCl and 1000 cc. N/10 NaOH from the N/2 standard solutions, and determine by titration whether they are equivalent.

8.—Determination of Nitrogen in Ammonium Sulphate

Ammonium sulphate for analytical work should be specially prepared[1], dried for one-half hour at 110 degrees C., and then allowed to stand in a dessicator over sulphuric acid until used.

a.—Preparation of a Standard Ammonium Sulphate Solution

Carefully clean and dry a small beaker of 100-150 cc. capacity. Place it on the left-hand pan of the balance, and accurately obtain its weight. Place in this beaker a sufficient amount of ammonium sulphate to weigh between 2.75 and 3.000 grams. Whatever the weight is, be sure to record it accurately to the fourth decimal place.

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[1]Folin and Farmer. Jour. Bio. Chem. 11, 406, 1912.

Clean the 500 cc. volumetric flask with cleaning fluid, wash out thoroughly with tap water, then rinse twice with small amounts of distilled water. Have a clean funnel also, which will fit conveniently into the neck of the volumetric flask.

Fill the beaker containing the ammonium sulphate two-thirds full of distilled water. Place on the wire gauze over a low flame, and stir gently until dissolved. *Do not lose a drop.*

Now transfer it carefully to the volumetric flask, without loss, pouring from the beaker by means of a stirring rod. Rinse the beaker and stirring rod 4 or 5 times with portions of distilled water.

To the flask add 1 to 2 cc. of concentrated hydrochloric acid, then fill the flask up to the neck with distilled water.

The flask has a capacity of 500 cc. only at a given temperature, namely: 15 degrees, or 20 degrees C. Note the temperature at which it was calibrated and proceed as directed in the preparation of the N/2 Oxalic Acid (page 6). While the ammonium sulphate is dissolving, it is a good plan to have the wash bottle immersed in a dish of cold water until the temperature required by the volumetric flask is obtained.

After the ammonium sulphate solution has stood sufficiently long to be thoroughly diffused, it should be transferred to a clean, dry bottle, and properly labelled. The label should not fail to show the concentration of the solution as well as the date on which it was prepared. This solution will be used in b below, and also later as directed on page 113.

b.—Distillation of Ammonia

Procure two clean 500-700 cc. Florence flasks.

Pipette 25 cc. N/10 hydrochloric acid into each.

Add 25 cc. distilled water.
2 drops of alizarin red.

Flasks so prepared can be depended upon to receive 30 milligrams of nitrogen. Place each flask on the shelf under the ammonia still. Insert the delivery tube, being careful that the end dips into the receiving acid.

Pipette 25 cc. of the standard ammonium sulphate solution prepared above, into each of two 800 cc. Kjeldahl flasks. Add a small pinch of powdered pumice, 400 cc. of distilled water

and two drops of alizarin red. Now place the flask upon the ring of the still. Just before stoppering add 2 to 5 cc. of concentrated sodium hydroxide, in such a manner that the alkali, when poured from the graduated cylinder, strikes against the neck of the flask 2 inches below that portion touched by the rubber stopper. The alkali, following along the inner wall of the flask, sinks to the bottom, and should be thoroughly mixed, *after the stopper is inserted,* by gentle rotation. *Caution*—Press stopper into neck of flask during mixing. Immediately light the burner below, regulate the flame so that gentle boiling ensues, and distill over about 300 cc. The delivery tube should be withdrawn into the neck of the flask while the last 100 cc. are coming over. Suction movements in the delivery tube can be reduced by tipping the receiving flask at such an angle that the acid just seals the opening of the tube. If in doubt, consult an instructor.

The distillation of ammonia is complete, when a drop of distillate, falling on a strip of neutral litmus paper, produces a color of no greater intensity than that produced by distilled water.

When the distillation is complete, disconnect the delivery tube and rinse into the flask by means of the wash bottle. Back titrate with N/10 sodium hydroxide and, from the data obtained, calculate the number of grams of nitrogen (or milligrams) in the ammonium sulphate taken. Check your results against the theoretical quantity, allowing an error of not over 0.5 milligram of nitrogen. Report your results to the instructor.

9.—Kjeldahl Determination of Nitrogen

Small test tubes containing uric acid have been placed in the drawer of each balance. This tube must be carefully wiped with filter paper and then placed upon the scale pan of the balance. Whenever the tube is to be handled, use filter paper to prevent adherence of grease from fingers. Record the weight of this tube to the fourth decimal.

Remove the tube from the scale pan and, while holding it over the neck of a clean 800 cc. Kjeldahl flask, remove the stopper. Carefully drop 50 to 60 milligrams of uric acid into the flask, then stopper the tube and re-weigh. The difference in weight should be recorded as the charge taken. Repeat this process with a second Kjeldahl flask.

To each flask add 15 cc. concentrated sulphuric acid (from a cylinder), using this acid to rinse down any uric acid adhering to the neck of the flask. Add 2 cc. of 5% copper sulphate, and finally a few small pieces of broken glass.

Place the flasks on the digestion rack in the hood, and light the burner. Allow the mixture to reach the boiling point then turn down the flame until boiling is just perceptible. *Avoid too vigorous boiling, otherwise a loss of nitrogen will occur.*

After one-half hour digestion, extinguish the flame and allow to cool. When cold, consult the instructor as to the manner of adding water to the acid in the flask. After the water has been added, add 2 drops of alizarin red, and then proceed to distill over the ammonia as directed under experiment 8-b.

Notice: The amount of alkali required for this distillation is greater than that indicated in the procedure above. Why? To calculate the alkali required, assume that 1 cc. of concentrated sulphuric acid is neutralized by 2.5 cc. of concentrated sodium hydroxide. See also note, page 108.

Calculate the nitrogen content of the uric acid taken, and check against the theoretical figure. Report your results to the instructor.

10.—The Law of Mass Action (Concentration Law)

Briefly stated, the Law of Mass Action reads: The rate of chemical change is proportional to the molecular concentration of the reacting substances. The rate of change is also under the influence of a "constant," which is characteristic of the chemical nature of the reacting substances and also of the temperature.

The following exercise is designed to illustrate:

(a) The change in speed of reaction with change in concentration of components (plotting of curve).

(b) The condition of equilibrium, i. e., where the speeds of reaction on the two sides have become identical.

(c) The manner in which the equilibrium constant can be calculated; and, finally,

(d) The effect of changing the concentration of one component upon the point of equilibrium of the reaction.

The reaction to be studied is that of the esterification of ethyl alcohol by acetic acid. To be successful, these directions must be followed minutely.

Equation, $C_2H_5OH + CH_3COOH \rightleftharpoons CH_3COOC_2H_5 + H_2O$.

Start, 1 mole + 1 mole O + O

Equilibrium, 1/3 mole + 1/3 mole $\rightleftharpoons$ 2/3 mole + 2/3 mole.

Procedure: Select a clean and dry 250 cc. Florence flask. Draw a reflux condenser from the stock room, and arrange flask and condenser, by means of suitable clamps, for refluxing. Place an evaporating dish, to serve as a water bath under the flask and support by a ring. The micro-burner should be used for heating.

When the apparatus is ready and known to be tight, take the flask to the stock room and get 29.26 cc. of absolute alcohol (half molar quantity). To this add from a Mohr pipette exactly 0.5 cc. of concentrated sulphuric acid. Add another 0.5 cc. of the acid to a small (150 cc.) clean beaker and set aside. The acid serves only to catalyse the reaction and influences the reaction otherwise to no appreciable extent.

Clean two pipettes, a 25 cc. and a Mohr; have both dry, Borrow a second Mohr pipette, having it likewise clean and dry. Place on the table a burette filled with N/2 sodium hydroxide. Place a second clean 150 cc. beaker on the table.

When all is ready, pipette exactly 28.35 cc. of glacial acetic acid into the Florence flask containing the alcohol. (Use the 25 cc. pipette and the 3.35 cc. from the Mohr pipette.)

Cautiously mix the components by gently rotating the flask, then immediately withdraw exactly 2 cc. of the mixture by means of the second Mohr pipette. Place this portion in the clean beaker; note the time by the watch (hour and minutes), then immediately attach flask to condenser and heat very gently. *Caution.* The temperature of the water bath is to be maintained at 70° C. by the thermometer. Note also that *only* two cubic centimeters of the mixture are withdrawn. Any excess in pipette should be immediately returned to the flask. Add 1 drop of phenolphthalein to mixture in beaker and titrate immediately with N/2 sodium hydroxide.

To the beaker containing the 0.5 cc. of concentrated sulphuric acid add 25 cc. of distilled water. Add 1 to 2 drops of phenolphthalein and titrate with N/2 sodium hydroxide. Divide the number of cc. of alkali required by 29 and subtract the quotient so obtained from each titration of 2 cc. of reaction mixture. This gives the correction figure for the sulphuric acid. After 10 minutes' heating, rapidly disconnect condenser, and withdraw another 2 cc. portion of the reaction mixture for titration in a similar manner as previously described. Stopper the flask (the condenser is never disconnected longer than necessary to remove 2 cc. sample, then continue heating. Repeat until 4 or 5 such titrations have been taken.

Allow the apparatus to remain connected with flame extinguished and cooling water turned off until another laboratory period if equilibrium has not been reached. Continue the experiment, making titrations, until two successive tests give the same titratable acidity. Record all values as shown in the table below.

When equilibrium is obtained, calculate the amount of reacting mixture which has been removed. Subtract this from the initial volume of mixture. Add a definite calculated amount of distilled water, then heat for 10 minutes, noting change in equilibrium. *Remember,* when calculating, the proportional decrease in titration due to dilution by water added! Continue until a new point of equilibrium is established, and check agreement with theoretical as deduced from reaction constant found.

Record data as below:

Time Minutes	Cc. N/2 NaOH	Correction (Factor for Acid)	Cc. N/2 NaOH (Corrected)
0.00			
10.00			
20.			
30.			
40.			
60.			
90.			
Etc.			
At Equilibrium			
After Addn. of Water 0.00			
10.00			
20.00			
30.00			
At Equilibrium			

Work out the following:

1. Plot the curve for the speed of reaction, putting time in hours along abscissa, and molecular change in concentration along ordinate.

2. Work out the value of the equilibrium constant "K."

3. Using the value found for K, add a definite molecular quantity of water, and calculate the molecular concentration of acid when equilibrium is again established. Check this value with that obtained by titration.

INDICATORS

The term "indicator" cannot be precisely defined. As used in acidimetry, an indicator is a weak organic acid, or the salt of such an acid, which manifests by a color change, differences in the reaction (hydrogen ion concentration) of the substance in which it is placed. The color change accompanying the change in reaction is frequently due to an intramolecular combination of a quinone group with a salt of phenol or analine, etc.

The student must clearly understand at the outset, that most indicators do not change color at the point of exact neutrality. The hydrogen ion concentration at which the change takes place is indicated in the tables on pages 544, Plate A, and 546, of Mathew's Physiological Chemistry, 3rd edition.

Strong and Weak Acids

When an acid is added to water, the acid is dissociated into the cation (H^+) and a corresponding anion. The completeness of dissociation at ordinary dilution is made the basis upon which its strength is judged. Acetic acid is dissociated but little, while hydrochloric acid is almost completely dissociated in N/10 solution.

A strong acid will remove the base from the salt of a weaker one. It is therefore possible to "neutralize an acid" by means of a salt as indicated in the following experiments.

11.—Replacement of Weak Acid by Strong Acid in Salts

a. Place 1 to 2 cc. of saturated sodium carbonate solution in a 250 cc. beaker.

Add 25 cc. distilled water.

Add 1 drop of methyl orange.

Add dilute sulphuric acid (1:4) until neutralized.

Notice the formation of gas. Explain and write equation.

b. Repeat the above experiment, placing 1 cc. of concentrated hydrochloric acid in the beaker, using congo-red as an indicator, and adding saturated sodium acetate solution. Explain and write the equation.

12.—Point of Change of Indicators

Select 10 clean test tubes of uniform size. Place the standard buffer solutions of pH values ranging from pH 1 to pH 10 in

these tubes. To each tube, add 1 drop of methyl orange solution (caution, previously diluted to one-fourth strength) and note point of change. Record observations.

Repeat the experiment with alizarin red, and phenolphthalein (diluted with 95% alcohol). Record.

The standard buffer solutions used are described on pages 543 and 544 of Mathews Physiological Chemistry, 3rd edition.

A more accurate set of standard buffer solutions is described by Clark and Lubs, Journal of Biological Chemistry, 25,479, 1916.

Preparation of Solutions of Definite pH value:

A. Stock Solutions—

1. Hydrochloric Acid—N/10, N/100, N/1000.
2. M/15 Potassium Dihydrogen Phosphate (KH_2PO_4).
   9.078 gms. KH_2PO_4 per liter.
   Designated as Primary Phosphate.
3. M/15 Disodium Phosphate (Na_2HPO_4).
   11.876 gms. Na_2HPO_4-2 H_2O per liter.
   Designated as Secondary Phosphate.
4. M/10 Secondary Sodium Citrate.
   Dissolve 21.008 gms. Citric Acid (Crystals) in 200.00 cc. N/10 NaOH.
   Dilute to 1000 cc.
   Designated as Citrate.
5. 0.2 M. Alkaline Borate.
   Dissolve 12.404 gms. Boric Acid in 100.00 cc. N/1 NaOH.
   Dilute to 1000 cc.
   Designated as Borate.

B. Preparation of Solutions of Known pH.—

Solution	Call	pH	Preparation
pH—1.04	"1"	10 cc.	N/10 HCl. (Students' Acid.)
pH—2.02	"2"	10 cc.	N/100 HCl. (Students' Acid.)
pH—3.01	"3"	10 cc.	N/1000 HCl. (Students' Acid.)
pH—3.95	"4"	5.50 cc.	Citrate plus 4.5 cc. N/10 HCl.
pH—4.96	"5"	10 cc.	Citrate.
pH—5.97	"6"	6 cc.	Citrate plus 4 cc. N/10 NaOH.
pH—6.98	"7"	6 cc.	Secondary Phosphate plus 4 cc. of Primary Phosphate.

pH—8.04 "8" 9.75 cc. Secondary Phosphate plus 0.25 cc. Primary Phosphate.
pH—9.01 "9" 8.5 cc. Borate plus 1.5 cc. N/10 HCl.
pH—9.97 "10" 6 cc. Borate plus 4 cc. N/10 NaOH.

Reference:

Sorensen, S.P.L. Biochem. Zeitschrift. 21, 167, 1909.
Michaelis, L. Die Wasserstoffionenkonzentration. Berlin. 1914.

The student is advised to consult the original literature respective to the theory underlying, and preparation of buffer solutions.

13.—Titration Experiments

Perform the following titrations, writing up your conclusions:

Place in Beaker	Add	Titrate with	Cc. with Methyl Orange	Cc. with Alizarin Red	Cc. with Phenolphthalein
10cc. N/10 HCl	15 cc. Dist. H_2O	N/10 NaOH			
10cc. M/10 H_3PO_4	15 cc. Dist. H_2O	N/10 NaOH			
10cc. M/10 NaH_2PO_4	15 cc. Dist. H_2O	N/10 NaOH			
10cc. M/30 Citric Acid	15 cc. Dist. H_2O	N/10 NaOH			
10cc. M/10 $NaHCO_3$	15 cc. Dist. H_2O	N/10 HCl			
10cc. N/10 NH_4OH	15 cc. Dist. H_2O	N/10 HCl			

If possible make a table showing the conditions governing the use of indicators (vide-Mathews, p. 868).

14.—Titratable Acidity and Reaction

Although two acids of like normality may neutralize the same quantity of alkali, their respective strengths (hydrogen ion concentration) will vary with their degrees of electrolytic dissociation. The following experiment is intended to make this relationship clear:

A. Equivalence of titration—because of same normality.

1. Pipette 10 cc. of N/10 hydrochloric acid into a beaker.

   Add 1 drop of phenolphthalein.
   Titrate with N/10 sodium hydroxide.
   Record the cubic centimeters required in the table below.

2. Repeat the above titration using 10 cc. of N/10 acetic acid, instead of the hydrochloric acid. Record the amount of alkali in the table 1.

B. Difference in strength—because of unequal degree of dissociation.

1. Select five clean test tubes of equal size. Into these, pipette respectively 10 cc. of the acids mentioned in the headings of the following table. To each tube, add 3 drops of a 0.1% solution of Methyl Violet 6B, and record the observed color in table 2.

Table 1.

Titration	10 cc. N/10 HCl	10 cc. N/10 CH_3 COOH
Cc. N/10 NaOH with Phenolphthalein		

Conclusions:

TABLE 2.

Titration	10 cc. N/1 CH_3 COOH	10 cc. N/10 HCl	10 cc. N/100 HCl	10 cc. N/1000 HCl
Color with 3 drops 0.1% Methyl Violet 6B				
pH				

Conclusions:

THE DETERMINATION OF THE HYDROGEN ION EXPONENT pH, BY THE GAS CHAIN METHOD

In 1909 Sörensen[1] proposed to indicate the reaction of a solution by the character pH, which he called the hydrogen ion exponent. It is the Brigg's logarithm of the reciprocal value of the normality of the solution, in terms of the hydrogen ion concentration.

The definition as well as the relationship between the hydrogen ion concentration $[H^+]$ and the hydrogen ion exponent will be made clear by the following examples:

Examples—

In N/10 HCl the $[H^+]$ is 0.091N

$$\text{then } 0.091\text{N} = 9.1 \times 10^{-2}$$
$$9.1 = 10^{X}$$
$$\log 9.1 = X \log 10 = X$$
$$X = \log 9.1$$
$$\text{in the log book } 9.1 = 0.96$$
$$\text{then } 9.1 \times 10^{-2} = 10^{0.96} \times 10^{-2} = 10^{-1.04}$$
$$\text{therefore pH} = 1.04$$

The above may be expressed by the following formula:

$$\text{pH} = \log \frac{1}{0.091} = \log 1 - \log 0.091$$
$$= 0.00 + (-2 + 0.96)$$
$$= 1.04$$

The student is advised to consult the table in Mathew's, page 544, 3rd edition, and, for practice, calculate pH values from $[H^+]$ values.

Ex.—	Concentration $[H^+] \times$ N.	Hydrogen Ion Exponent pH
Given	5.76×10^{-4}	?

[1]Sörensen, Biochem. Zeitsch. 21, 159, 1909.

From your observations in a previous experiment fill in the proper pH values.

From this experiment, what is the explanation of "b," page 19?

Write one page summarizing your knowledge of indicators. Include specific statements drawn from the experiments in this section.

This means that log 5.76 is an exponent of 10

$$\log 5.76 = 0.76042 = 10^{0.76042}$$

Then $10^{0.76} \times 10^{-4} = 10^{-3.24}$

Therefore pH = 3.24.

To change hydrogen ion exponent to concentration, proceed in the following manner:

Example—

Hydrogen Ion Exponent	Concentration
	$+$
pH	$[H] \times N.$
Given: 5.05	?

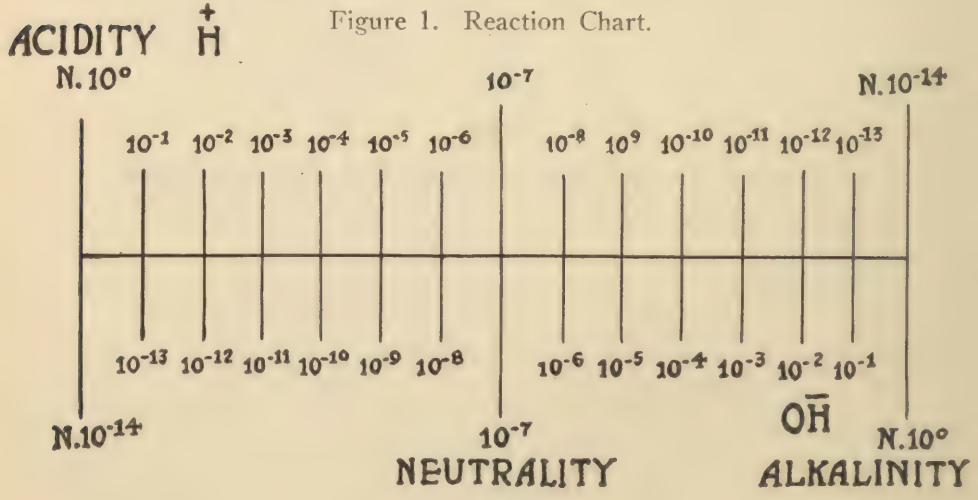
This means that pH 5.05 is $10^{-5.05}$

Then $10^{-5.05} = 10^{-6} \times 10^{+0.95}$

In the log book Mantissa 0.95 corresponds to number 8.9125, therefore pH $5.05 = 8.9 \times 10^{-6}$ N.

The following diagram, Fig. 1, will help the student to understand the method of recording an alkaline solution in terms of hydrogen ion concentration.

Figure 1. Reaction Chart.



The Gas Chain Apparatus

The simple gas chain apparatus[1] used by students in this laboratory is shown in Figure 2.

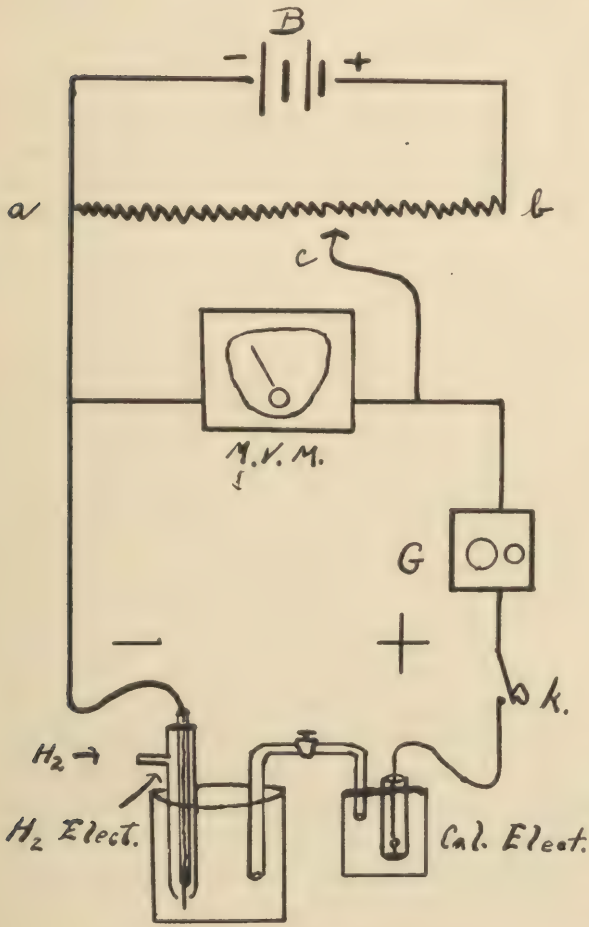


Figure 2. The Gas Chain Apparatus.

It consists of a two-cell Edison storage battery B, the terminals of which are attached to the high-resistance wire a-b, thus allowing a uniform drop in potential The negative wire continues to the hydrogen electrode. A sliding contact (c) taps off any desired fraction of the potential, which is indicated in millivolts by the millivolt meter shunted across the circuit. G is a simple galvanometer to indicate the nul point, when key k is pressed. A saturated calomel electrode is attached at the end of the positive wire, while the negative wire terminates in the platinum wire of the hydrogen electrode.

[1] The student is advised to read—Hildebrand, Jour. Am. Chem. Soc. 35, 846, 1913.

For the preparation of the Calomel electrode see Findlay, Practical Physical Chemistry. For the Hydrogen electrode, see Bovie, Jour. Med. Research, 33, 295, 1915.

In using the apparatus the determination is made briefly as follows: The solution to be tested is placed in the beaker. The platinum wire of the hydrogen electrode, previously covered with platinum black, is immersed as shown. The capillary tubing from the saturated calomel electrode, which is filled with saturated potassium chloride solution and prevented from syphoning by the closed, ungreased stop-cock, is then dipped into the liquid.

Hydrogen gas is now bubbled through the solution for a minute or two, until the electrode is saturated. A bubble should remain around the upper part of the platinum wire in the neck of the jacket tube after the flow has been shut off to keep the electrode completely saturated. The gas is washed by passing through an alkaline pyrogallic acid solution to remove traces of oxygen if present, then through a saturated mercuric chloride solution, to remove sulphides, and finally through distilled water.

After the apparatus has stood for a minute or two the galvanometer is watched while the key is quickly closed and immediately released. If the needle moves, the circuits are not in balance. Sliding contact c is now adjusted and the key again closed as above. The contact is moved and the above process repeated until the circuits are in balance.

The combined voltage of the calomel electrode and that generated by the ions about the hydrogen electrode has now just balanced the imposed potential of the battery current, consequently its value is indicated by the millivolt meter. Note: The mercury at the bottom of the calomel electrode is always the positive element, while the platinum wire of the hydrogen electrode is the negative. These, when joined by the capillary tube containing potassium chloride solution, form a small battery. The circuits are so connected that the current from this "battery" opposes in direction of flow that supplied from the storage cells.

Calculations

The hydrogen ion exponent pH is calculated according to the following equation:

$$pH = \log \frac{1}{[H]} = \frac{\text{E. M. F. (Obs. in volts)} - \text{E. (Cal. Elec. in volts)}}{0.0001983\ T}$$

Note: T is the absolute temperature.

The values for E are given below in millivolts.

Temperature	N/10 Cal. Elect.	Satd. Cal. Elect.
15° C.	.....	252.5
16° C.	.....	251.7
17° C.	.....	250.9
18° C.	337.7	250.3
19° C.	.....	249.5
20° C.	337.5	248.8
22° C.	.....	247.5
24° C.	.....	246.8
25° C.	.....	246.3
30° C.	336.4	....
37° C.	.....	235.5
38° C.	335.5	235.0
40° C.	333.9	....
50° C.	332.6	....
60° C.	329.0	....

If all voltages are recorded in millivolts, the expression for the temperature correction (0.0001983 T) has the following value at the temperature indicated in Centigrade degrees:

15° C.....	57.1	28° C.....	59.7
16° C.....	57.3	29° C.....	59.9
17° C.....	57.5	30° C.....	60.07
18° C.....	57.7	31° C.....	60.27
19° C.....	57.9	32° C.....	60.47
20° C.....	58.1	33° C.....	60.66
21° C.....	58.3	34° C.....	60.86
22° C.....	58.5	35° C.....	61.06
23° C.....	58.7	36° C.....	61.25
24° C.....	58.9	37° C.....	61.45
25° C.....	59.1	38° C.....	61.64
26° C.....	59.3	39° C.....	61.85
27° C.....	59.5	40° C.....	62.05

Testing of Calomel Electrodes: Before using a new electrode its E. M. F. should be tested against a hydrogen electrode, using a standard fluid of known value. The best solution for

this purpose is the "Standard Acetate Mixture," recommended by Michaelis. It is prepared as follows:

Normal sodium hydroxide	50 Ccm.
Normal acetic acid	100 Ccm.
Distilled water	350 Ccm.

When testing with the above solution, values agreeing within $\pm$ 0.5 millivolt with those recorded below should be obtained:

Temperature	Against N/10 Elect.	Against Sat'd. Elect.
15° C.	602.5 millivolts	517 millivolts
16° C.	.....	.....
17° C.	.....	517 millivolts
18° C.	604.5 millivolts	517 millivolts
19° C.	.....	518 millivolts
20° C.	.....	.....
21° C.	607.5 millivolts	518 millivolts
22° C.	.....	518 millivolts
23° C.	.....	519 millivolts
24° C.	.....	.....
38° C.	.....	520 millivolts

The above potentials should be obtained after 1 hour, and will remain constant for at least 24 hours. At first a lower voltage is found due to the rapid diffusion occurring.

The student is required to become familiar with the theory of the gas chain apparatus, and then do the following experiments:

15.—Determination of pH Value

1. Determine the pH value of your N/10 HCl solution. How does it compare with the value given on page 21?

2. Determine the pH value of the N/1 CH_3COOH solution used in experiment 14, page 19. Now fill in the value required in table 2, page 20.

16.—Electrometric Titration

1. Place 10 cc. of N/2 HCl in a beaker. Add sufficient water to submerge the hydrogen electrode to the required depth. Place your N/2 NaOH in the burette. Determine the pH value

of the acid, then add about 1 cc. (recording the exact amount) of the N/2 NaOH and again determine the pH value. Repeat this process until 9 cc. have been added. Now add 0.25 cc. portions until 2 cc. more have been added, then finish by adding 2 cc. portions until a total of 16 cc. have been added.

Obtain a piece of plotting paper. Plot pH values along the ordinate and cubic centimeters of alkali along the abscissa. Note the point at which your titration curve cuts the pH 7 line. The cubic centimeters of alkali equivalent to this on the abscissa is the quantity required for the neutralization of the acid.

2. Repeat the above titration using 5 cc. of 0.5 molar phosphoric acid, adding the alkali slowly (about 0.25-0.5 cc. portions), when the voltage is rapidly rising. Add a total of 16 cc. of N/2 NaOH.

Plot your curve and compare with that previously obtained for hydrochloric acid.

3. Repeat the titration, using 10 cc. N/2 acetic acid, adding a total of 16 cc. N/2 NaOH. Plot and compare the curve with those previously obtained.

4. Write about a page, stating how you would prepare a normal phosphoic acid solution. Be specific in stating your reasons.

LIPINS

The term Lipin is used here to indicate a group of substances which may be extracted from plant or animal tissue by alcohol and ether (Rosenbloom, 1911). Some of these substances are true fats, that is, esters of glycerol with higher fatty acids. Others, as for example, the essential oils, waxes, and sterols, "bear no resemblance whatever to fat, except the accidental one of being soluble in the ordinary fat solvents." (MacLean, 1918.)

The following is a rough classification of the material extractable from tissue by alcohol or ether:

I.—Fats

A. Neutral Fats. The esters of glycerol with higher fatty acids frequently accompanied by fatty acids. The latter are formed through the action of lipases in the tissues upon neutral fats.

II.—The Fat-Like Bodies

B. The Phosphatides (Thudichum). These substances contain fatty acid, nitrogen and phosphorus.

1. Monamino-mono-phosphatides, N :P : :1 :1,
   Examples, Lecithin and Kephalin.
2. Diamino-mono-phosphatides, N :P : :2 :1,
   Example, Sphingomyelin.
3. Monamino-diphosphatides, N :P : :1 :2,
   Example, Cuorin.

C. The Cerebrosides (Thudichum). These substances contain fatty acid, nitrogen, and a carbohydrate group. They do *not contain phosphorus.* The carbohydrate is lactose.

Examples, Phrenosin, and kerasin.

III.—Unrelated Substances

D. Waxes. These substances are esters of sterols (monohydric alcohols, solid frequently and often terpenes) and higher fatty acids. Examples, Sperm oil, bees wax.

E. Sterols. These substances are alcohols, mostly terpenes, which are solid at room temperatures . They are therefore chemically unrelated to true fat. Examples, cholesterol, and phytosterol.

17.—Solubility of Neutral Fat

A. Put a small piece of beef suet in each of four test tubes. Add 5 cc. water, alcohol, ether, and choloroform to the respective tubes. Shake. Do you notice solubility in any of the solvents?

B. Put a small piece of beef tallow in each of four test tubes. Add solvents as directed above. Record result.

Note: Suet is natural tissue fat as it occurs in the animal. Tallow, on the other hand, is the fat from tissue, tried out by heat, therefore devoid of cellular structure.

18.—Emulsification of Fats

When a substance goes into solution, the result is a fluid of perfect homogenity. Immiscible fluids like oil and water, when mixed with soap, proteins, gums or the physiological fluid bile, may, by agitation, become more or less permanently subdivided into minute particles. This state is known as an emulsion. One of the characteristic properties of this state is the development of an extremely great surface area, as compared with the mass of particles. The following experiments are intended to bring out the above-mentioned facts.

Prepare a series of test tubes containing the following:

Tube	Oil Added	Other Addition	State After	
			Shaking	30-min. Standing
1	3 drops neutral cotton seed oil	5 cc. water		
2	3 drops neutral oil	5 cc. of 0.25% Na_2CO_3		
3	3 drops rancid oil	5 cc. water		
4	3 drops rancid oil	5 cc. of 0.25% Na_2CO_3		
5	3 drops neutral oil	5 cc. of 2% egg albumen		
6	3 drops neutral oil	5 cc. of 10% gum arabic		
7	3 drops neutral oil	4 cc. water plus 1 cc. bile		

Shake all tubes thoroughly and equally. Note the appearance immediately after shaking and again after 30 minutes' standing. Why do the particles in an emulsion not cohere, and form stratified layers? Examine one of the emulsions under the microscope.

19.—Fat Crystals

Fat from different sources may be made to crystalize in definite and characteristic form which may assist in its identification. Usually fat crystals are transparent, and therefore overlooked. The addition of Sudan III to the solution of fat, makes their detection extremely easy.

Place a piece of fresh, dry tallow (beef, mutton, etc.) about the size of a pea in a dry test-tube. Add dry chloroform drop by drop until the tallow is dissolved. Assist the solution of the tallow by holding the tube near, *but not in* a flame. When dissolved, allow to cool to room temperature and add a few drops more chloroform if the fat separates out.

To this saturated solution of fat, add three volumes of chloroform and enough Sudan III from the point of a knife blade to color the solution fairly heavily. Do not add so much dyestuff that particles remain undissolved. Note: To save the dyestuff, a sufficient amount has been added to the chloroform placed on the shelf for this experiment.

Clean a microscope slide and also a square cover slip. By means of a pipette, place 3 drops of the fat solution in the center of the slide. Cover immediately with the cover slip, and watch for the formation of characteristic crystals at the edge of the cover slip as the solvent slowly evaporates.

Larger crystals may be obtained if one gram of tallow is dissolved in 10 cc. chloroform and stained as directed above. The "bunching" of the crystals varies somewhat with the concentration of the fat.

Sketch the crystal obtained, and, if possible, make preparations from three or four different fats and also from fatty acids. Sketch and label.

20.—The Iodine Number—Wij's Method

Certain fats and oils contain varying amounts of the esters of unsaturated fatty acids. It is possible to cause iodine to enter the molecule at these points of unsaturation (double bonds). The iodine number serves as an index of the relative degree of unsaturation of fats and oils.

Start simultaneously the determination of the iodine number of two fats (beef tallow and some one of the oils).

Weigh out about 0.3 gm. of oil or about 1 gm. of beef tallow into a 250 cc. Florence flask, and dissolve in 10 cc. chloroform. Add 25 cc. Wij's iodine solution by means of a pipette; stopper, and put in a dark place for one-half hour. Add 15 cc. of 10% potassium iodide and dilute with 100 cc. of water, then titrate the excess of iodine (partly in solution in water and partly in the chloroform) with N/10 Sodium thiosulphate, running the latter into the flask until after repeated shaking, both the chloroform and the watery solution are but faintly straw colored. Then add 1 cc. of 1% starch solution and continue the titration to the disappearance of the blue color.

While waiting for the absorption of the iodine to take place, the value of the iodine solution may be determined in terms

of N/10 sodium thiosulphate, by adding to 25 cc. of Wij's solution, 15 cc. of 10% potassium iodide and titrating as above. The difference between the two values represents the amount of iodine absorbed by the fat, and is calculated in terms of *grams of iodine per 100 grams of fat.*

Example: Charge is 0.3 gm. cottonseed oil.
Back titration is 35 cc. N/10 $Na_2S_2O_3$.
Wij's solution, 25 cc. equals 60 cc. N/10 $Na_2S_2O_3$.
Therefore, 60 – 35 equals 25 cc. N/10 Iodine.
Since 1 cc. N/10 Iodine = 0.0127 gm. we have,
$25 \times 0.0127 = 0.317$ gm. Iodine absorbed.
Therefore $\frac{100}{0.3} \times 0.317 = 105.6$.
Iodine number is 105.6.

Wij's Iodine Solution

Dissolve 13 grams of iodine in 1 liter of glacial acetic acid. Titrate the iodine content of the solution, then pass washed and dried chlorine gas into the solution until the titration number is doubled. A very distinct change in color of the solution indicates when this has taken place.

The relative value of the Wij's iodine solution to N/10 sodium thiosulphate will be given by the instructor.

Using oleic acid as an example, write structurally the reaction which would take place in this determination.

Write the structural formulae of three other fatty acids which would have a high iodine number.

21.—The Saponification Number (Köttstorfer Value)

The saponification number is the number of milligrams of potassium hydroxide required to completely saponify one gram of fat or wax. Since during saponification, the neutral glycerides or other esters (waxes) of fatty acids are decomposed, yielding the potassium salt of the fatty acid (soap) and the corresponding alcohol (glycerol from neutral fats), it is apparent that the lower the molecular weight of the fatty acids involved, the greater the saponification number.

Procedure: Procure two clean, dry Erlenmeyer flasks of 200-250 cc. capacity. Into one of these weigh 1.5 to 2 gms. of purified and filtered fat. Pipette into *each* 25 cc. of special alcoholic potassium hydroxide solution. To each attach an efficient condenser, and heat for one-half hour on the boiling water bath. (The heat should be so regulated that the alcohol is just simmering.) *Caution*: Avoid loss of alcohol through volatilization from condenser. The flasks are gently shaken in a rotatory direction at frequent intervals to thoroughly mix the contents.

After one-half hour, remove the flasks from the water bath, cool to room temperature, add 1 cc. of 1% phenolphthalein, and then titrate each with N/2 hydrochloric acid.

From the titration of the blank, subtract the titer of the flask to which fat was added. The difference corresponds to the quantity of N/2 potassium hydroxide solution required to saponify the weight of fat taken. This, when calculated to milligrams of potassium hydroxide per gram of fat, is the saponification number.

Potassium hydroxide in alcoholic solution turns dark brown on standing and especially if exposed to the light unless the alcohol used as solvent has been previously purified. The most satisfactory method we have found is to mix the ordinary 95% alcohol with commercial granular caustic soda; about 200 grams to 5 gallons of alcohol; and allow the mixture to stand in the light for several weeks. The alcohol is then distilled off. The distillate is neutral, and when used in preparing an alcoholic solution of potassium hydroxide, the reagent remains clear practically indefinitely.

To prepare the special potassium hydroxide solution, weigh out 40 grams of "Potassium Hydroxide C. P. by alcohol," dissolve in the purified alcohol, finally making the volume up to 1 liter. If a precipitate of carbonate is noticed, it is removed by filtering the reagent through glass wool.

Problem: Calculate the saponification numbers of ethyl acetate and of glyceryl tributyrate, respectively.

22.—Fat in Milk (Babcock's Method)

The physician is frequently called upon to modify cow's milk for infant feeding, as well as to analyze breast milk to determine the cause of malnutrition in infancy. Since the fat

content in both cases is of primary importance, the following methods are given.

A. Cow's Milk.

Procure a Babcock bottle, graduated to 10% butter fats. Into this pipette 17.6 cc. (18 grams) of milk. Add 17.5 cc. of commercial sulphuric acid (sp. gr. 1.82-1.84). Mix by rotation until all curds are dissolved. The mixture becomes hot and dark colored.

The bottle is now placed in the bucket of a Babcock centrifuge, and counter-balanced with a duplicate test bottle, or by other suitable means.

Revolve in the centrifuge for 5 mins. at a rate of 800 to 1,000 revolutions per min. Stop the machine, remove the bottle then add boiling water until the liquid comes *just to* (but not into) the graduated neck. Return to the centrifuge, and revolve for 2 mins. Again stop the centrifuge, remove the bottle, add boiling water until the graduated neck is nearly filled. Return to the centrifuge and revolve again for 2 minutes. The bottle is calibrated to give directly the percentage of fat in the sample by noting upper and lower readings of fat column, provided the temperature is 60° C. It is therefore best to immerse the bottle nearly to the top of the neck in a beaker of water heated to 60° C., for two minutes before making the readings.

B.—Human Milk

For human milk, where usually the amount available is limited, a special bottle which requires but 5 cc. is used. This bottle fits the cup of an ordinary 15 cc. centrifuge tube used for urine sediments.

Procedure: Fill the special bottle with milk to the 5 cc. mark. Add commercial sulphuric acid until the body of the bottle is filled. Rotate until curds are dissolved. Fill the neck of the bottle with a mixture consisting of equal parts of concentrated hydrochloric acid and amyl alcohol.

The tube is next placed in the cup of the centrifuge, counter-balanced, then rotated for 2 minutes. The percentage of fat is now read by observing its location in the graduated neck of the bottle.

23.—Refractive Index of Butter Using Zeiss Butero-Refractometer

The determination of the refractive index of fat or oil serves its greatest purpose in the detection of adulterants. It also assists materially in identification of pure fats and oils.

The work in the laboratory is intended to be upon butter. Any other fat or oil having a refractive index within the range of the instrument (1.4220 to 1.4895) may be substituted.

Open the movable half of the prism block of the refractometer and clean all surfaces thoroughly and carefully with a piece of fine linen cloth moistened with a little alcohol or benzine.

See that the temperature of the heating system is constant and a few degrees above the melting point of the fat under investigation. Holding the instrument so that the open half of the prism is horizontal, carefully applying a drop or two of the clear (filtered, if necessary) melted fat or oil to its optical surface. Close the prism faces and lock by turning the thumb-screw at the top of the movable half of the prism.

Adjust the mirror so that the critical line separating the bright left-hand field from the dark right is clearly visible when looked at through the telescope. If the critical line is not distinct probably the space between the prism faces is not uniformly filled by the oil or fat. Finally adjust the movable part of the ocular so that the scale becomes clearly visible.

Allow a current of water at a proper and uniform temperature to flow through the instrument for some time previous to taking the reading. Notice the critical line. If hazy at first, it should, after a minute, attain a fixed position and reach maximum distinctness.

When this is attained, notice whether the critical line is colorless or colored, and if the latter, what color. Now note the exact location of the critical line on the centesimal scale, estimating to tenths of a division by means of the milled scale head on the side of the instrument. Immediately read the temperature recorded by the thermometer.

This instrument is primarily intended for use with butterfats, which, if pure, give a colorless critical line. A red colored line indicates low dispersive power, while a blue line is given by a substance of relatively high dispersion. Oleomargarine shows a greater dispersion, and therefore a blue colored line.

After the instrument has been read, a table should be consulted for temperature correction and also for transposing the scale readings to indices of refraction (nD). Such tables may be found in most books on food analysis, e. g., Leach—"Food Inspection and Analysis," 3rd Ed., P. 105.

Use of Special Thermometer. Instead of employing the ordinary thermometer a special instrument has been devised for work with butter and lard. It has two scales, the left for butter, the right for lard, each of which indicates at once the highest allowable reading for the pure fat, corresponding to the temperature at which the observation is being made.

If the scale reading of the instrument as observed through the telescope, differs materially from the reading of the special thermometer, the fat under examination is undoubtedly adulterated, or in the case of butter, a higher reading indicates oleomargarine. The special thermometer thus indicates the highest possible number for pure butter.

24.—Saponification of Neutral Fat by Pancreatic Lipase

Dilute 1 part of 16% cream with three parts of water. Pipette 10 cc. of this fat suspension into each of two small flasks (100-150 cc.) or beakers. Pipette a third portion of 10 cc. into a 250 cc. Erlenmeyer flask. Saponify this sample as directed under Exp. 21 above, using only 10 cc. special alcoholic potash, recording your value in cc. of N/2 alkali required by the 10 cc. portion.

Caution: Be sure that the diluted cream suspension (emulsion) is uniform as each sample is pipetted out!

To one of the small flasks, add 2 drops of chloroform, and 2 cc. of distilled water. Cover the top with a tinfoil cap. Label with your name and the word "control." To the other flask is added 2 drops of chloroform, 1 cc. of distilled water and 1 cc. of freshly prepared 10% pancreatin suspension (Fairchild's Holadin).

Cover this flask with a tinfoil cap, and label properly. Place both flasks in the incubator at 40° C.

At the next laboratory period, not less than two days, remove the flasks, record any difference in appearance, then add 1 cc. of 1% phenolphthalein and 10 cc. of 95% alcohol to each.

Carefully titrate each flask with half normal sodium hydroxide. Notice the appearance of soap suds on one of the flasks you titrate. Explain. Subtract the titre of the "control" from that of the flask to which pancreatin was added. The difference gives the amount of alkali required to neutralize the fatty acids formed by the hydrolytic cleavage of the butter fats. (The cream is assumed to be fairly free from protein. No allowance is made for amino acids.) From this value, and that obtained by saponification with alcoholic potash, calculate the approximate percentage digestion effected by the pancreatic lipase.

25.—Saponification of Tallow. Preparation of a Soap

Preparation of a Fatty Acid. Detection of Glycerol.

Place 20 grams of tallow in a 6-inch metal dish. Add 30 cc. of 10 per cent sodium hydroxide (in 70 per cent alcohol) solution, and heat upon the steam bath until dry. Add 150 cc. of boiling water to the dish and stir until all of the soap is dissolved. Divide the solution into two equal parts, and proceed with each as follows:

A. Preparation of a Soap. Heat one portion of the soap solution to boiling. Add about 10 grams of sodium chloride, stir until dissolved, then allow the separated soap curd to collect in a cake as the solution cools. When cold, remove the soap. Try its solubility in water, alcohol, etc. To a portion of soap dissolved in water add a few cubic centimeters of 2% calcium chloride solution. Examine the curd; observe its greasy feel.

Write the equation for the saponification of the tallow (stearin). Write an explanation of the action of the sodium chloride upon the soap solution, using equations wherever possible. What soaps are soluble? What is the cause of the difficulty encountered in using soaps in a limestone country?

B. Preparation and Properties of Fatty Acid. Heat the second portion of the soap solution to boiling. Add 5 drops of methyl orange, and then with constant stirring, add dilute (1:3) sulfuric acid until a distinct acidity is obtained, and the liberated fatty acid floats on the surface as an oily layer. Filter the hot (but not boiling) mixture through a large dry filter paper, collecting and saving the filtrate for part "C" below. Wash the fatty acid on the paper with a small amount of hot water, then allow to cool. When cold, note its physical appearance; try its

solubility in water, alcohol, ether, etc. Dissolve a little fatty acid in alcohol, add a drop of phenolphthalein, and try to titrate with N/10 NaOH. What is formed? Dissolve another portion of fatty acid in alcohol and add to it a dilute (1%) sodium carbonate solution. What is formed? Write equations. Compare the above with the behavior of neutral fat and soaps when treated in a similar manner. If in doubt *try each reaction.* (Note: When neutral fat is to be dissolved in alcohol, the tube should be stood in warm water, *never held in a flame.*)

C. Isolation and Detection of Glycerol. To the filtrate from the preparation of fatty acid ("B" above) add 20% sodium carbonate solution, drop by drop while stirring until any acid present is just neutralized. Pour the solution into a 6-inch dish and evaporate on the steam bath to dryness. When dry, extract the residue with two 25 cc. portions of 95% alcohol. Combine the alcoholic extracts, pour into a small evaporating dish and evaporate off the alcohol, placing the dish upon the top of the steam bath. The material remaining should be crude glycerol.

Perform the following tests with the glycerol: Cautiously note taste. Note solubility of a drop in ether.

Borax Bead Test: Moisten a platinum loop with glycerol. Touch it to a little powdered borax, allowing some to adhere. Fuse in a bunsen flame, noticing the characteristic green color imparted to the flame. This color is due to the formation of a glycerol ester of boric acid.

Acrolein Test: Moisten about 2 grams of potassium bisulphate, contained in a small metal spoon, with 3 or 4 drops of the crude glycerol. Heat the spoon over a bunsen flame until dense white fumes are emitted. Remove from the flame and *cautiously* note the characteristic sharp stinging odor. Note also effect upon the eyes.

Glycerol loses hydrogen and hydroxyl groups equivalent to two molecules of water during the above heating. Rearrangement of the molecule occurs, forming acrylic aldehyde (acrolein). Write structural formulae indicating the reaction.

Repeat the above tests with any neutral fat. Explain. Do fatty acids or soaps respond to this test? Do waxes? If in doubt, try each.

26.—Exercise

Identification of Neutral Fat, Fatty Acid, and Soap, when occurring in a mixture.

The solubilities and reactions given above should enable the student, after a little thought, to identify fat, fatty acid, or soap, when occurring separately or in a mixture containing any two or all three.

Make out a scheme of procedure for this purpose, and then obtain two unknowns from the instructor. Report your findings on a slip of paper, dated and signed.

27.—Lecithin Preparation from Egg Yolk

(Procedure from Manual of W. Lee Lewis.)

1. Place 2 freshly separated egg yolks in a 300 cc. Erlenmeyer flask. Stopper and shake.

2. Add 100 cc. of 95% alcohol, then stopper and shake for two minutes. Now stopper flask with cork, through which passes a 4-foot glass tube (8-10 cm. in dia.).

3. *Boil gently* for 45 mins. on the steam bath.

4. Decant alcohol through dry filter paper into a dry 500 cc. flask. *Stopper.*

5. Add to residue in flask 100 cc. 95% alcohol, repeat extraction, and decant as above. (Nos. 2, 3 and 4.)

6. Add to residue in flask 100 cc. ether, then extract, and repeat a second time, exactly as directed above (No. 3, No. 4). (Caution—Place flask on cool part of steam bath top—ether must not be allowed to boil out.)

7. Evaporate united alcohol-ether extracts in a large evaporating dish on the water-bath until solvent is gone. *Caution—avoid flames!*

The residue in the evaporating dish contains the egg fat together with impure lecithin and cholesterol.

8. Extract the residue, by means of a glass stirring rod, with 3 portions of cold ether, using 20 cc. each time. (Filter if decantation is difficult.)

9. Add acetone to clear ethereal solution, *little by little,* until a maximum precipitate occurs. This is impure lecithin.

10. Allow precipitate to settle, decant and *save* supernatant liquid, collect lecithin on a glass rod; transfer to a watch glass.

11. Dry in a dessicator. (Keep lecithin in dessicator until collected.)

12. Perform the following tests:

Physical and Chemical Properties

a. Emulsification. Shake a small piece of lecithin with 5 cc. of water. Note the emulsion formed. Note myeline movements when a drop on a slide is examined under the microscope.

b. Presence of phosphorus. Fuse another small portion with three parts of fusion mixture (one part of potassium nitrate and two parts of sodium carbonate) in the spoon. When the hot mixture is white, allow to cool, dissolve in a small amount of water and test for phosphorus by means of nitric acid and ammonium molybdate solution. If the yellow precipitate does not form at once, warm and rub the sides of the test tube with a glass rod. What is the precipitate?

c. Saponification Test. Heat another fraction of your preparation with 15 cc. of sodium alcoholate in a flask for 10 minutes; evaporate to dryness. What is the residue? Dissolve in some hot water, acidify with sulphuric acid and allow to stand over night. Notice the separation of fatty acids.

d. Glycerine. Fuse half of the remainder of your preparation with two parts of potassium bisulphate.

e. Test for Nitrogen. Form a paste from the remainder of the lecithin with three parts of slaked lime and a few drops of water in the bottom of a small beaker. Cover the beaker with a watch glass on the lower side of which a moist piece of red litmus paper has been made to adhere. Now heat the beaker and contents very gently. Notice the change in color of the litmus. Explain.

28.—*Cholesterol* Preparation from Egg Yolk.

(Procedure from the manual of W. Lee Lewis.)

1. Evaporate ether-acetone solution from the previous experiment to dryness on the water bath. (Use evaporating dish.)

2. Saponify fats by adding 50 cc. of N/1 alcoholic sodium hydroxide, which dissolves residue. Cover evaporating dish loosely with a watch glass, and heat on the water bath until all of the alcohol has evaporated. (Use top of water bath.)

3. Transfer dry residue to a mortar and grind finely.

4. Extract with three 15 cc. portions of cold ether, triturating thoroughly.

5. Filter ethereal extracts, into a small beaker.

6. Allow slow spontaneous evaporation of the ether to occur on the desk, until crystals are formed.

7. Filter crystals from mother liquid (after latter is nearly evaporated).

8. Recrystallize from 95% alcohol. Filter and dry.

9. Sketch crystals and perform the following tests.

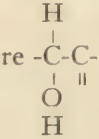
a. Evaporate a few of the crystals with concentrated nitric acid to dryness. Observe the color of the residue and add a few drops of ammonia. Notice the change of color from yellow to brick red.

b. Salkowski's reaction. Dissolve a crystal or two in one cc. of chloroform and add an equal volume of conc. sulphuric acid. Notice violet coloration of chloroform due to certain hydrocarbons formed by dehydration.

c. Schiff's reaction. Evaporate a few crystals of your preparation to dryness with two volume of concentrated sulphuric acid and one volume of ferric chloride solution. Notice violet color.

CARBOHYDRATES

Carbohydrates are aldehyde or ketone derivatives of polyhydric alcohols; or polymeric forms of these, yielding active aldehydo-alcohols, or keto-alcohols upon hydrolysis.

The simple carbohydrates contain one carbonyl group and one or more hydroxyl groups. It is essential that one of the hydroxyl groups be linked to a carbon, which is in direct union with a carbonyl group. The characteristic group of these compounds is therefore 

Classification:

Carbohydrates

1. Monosaccharides (2-7 carbon atoms).
2. Disaccharides (12 carbon atoms).
3. Polysaccharides (18 carbon atoms and above).

For a complete classification of carbohydrates the student is expected to refer to Mathews, p. 19.

29.—Elementary Composition—

Tests for Carbon and Hydrogen.

Place a little sugar in the bottom of a clean, dry test tube, and heat gently over the micro burner. Notice the changes that are taking place. Into the mouth of the test tube thrust a stirring rod moistened with barium hydrate solution. What is formed? Notice drops of moisture collecting on the sides of the test tube. To what are they due?

30.—Molisch-Udransky Test

Place 5 cc. of concentrated sulphuric acid in a test tube. Incline the tube and slowly pour down its inner side 5 cc. of 0.5% glucose solution, to which 3 drops of Molish's reagent (1% alpha napthol in alcohol) has been added. The sugar solution should stratify over the sulphuric acid. A reddish-violet zone is produced at the point of contact. This reaction is due to the formation of furfurol from the carbohydrate by the action of concentrated sulphuric acid. The furfurol then condenses with alpha napthol. This test is given by all bodies containing a car-

bohydrate group, whether free or in combination, as in certain proteins. It is therefore not specific for the class of compounds we designate by the name "carbohydrates."

Monoses or Monosaccharides

All monosaccharides and certain disaccharides give the following four characteristic tests:

1. Formation of silver mirror—by warming a tube containing ammoniacal silver nitrate to which a monosaccharide has been added.

2. Formation of caramel and resins—by warming a monosaccharide to which an alkali has been added.

3. Reduction of an alkaline copper solution—when an alkaline copper solution to which a monosaccharide has been added, is heated; reduction is evidenced by the formation of a yellowish-red suboxide of copper.

4. Formation of an osazone—when monosaccharides are heated with an excess of freshly recrystallized phenyl-hydrazine, in the presence of a dilute acid, characteristic yellow needle-shaped crystals are formed. The melting point of the crystals serves to identify the mono-saccharide. (This test is given by all reducing carbohydrate.)

31.—Silver Mirror

Procure a small test tube. Have it perfectly clean inside, then add 5 cc. of an ammoniacal silver nitrate solution. Add 8-10 drops of 0.5% glucose solution and place the tube in a beaker of boiling water. A bright silver mirror should be formed, indicating reduction of the reagent by the glucose.

32.—Moore's Test

To 5 cc. of dextrose solution in a test tube, add 5 cc. of 5% sodium hydroxide solution, and warm. The solution becomes first yellow, then orange, yellowish brown, and lastly, dark brown. Notice a faint odor of caramel. This odor is more pronounced upon acidification.

33.—Trommer's Test

To 5 cc. of a 1% copper sulphate solution in a test tube, add an equal volume of 5% sodium hydroxide solution. Note the formation of a precipitate and its disappearance. Boil. Note the result and compare with the following:

Trommer's test is based upon the property that dextrose possesses of reducing cupric hydroxide in alkaline solution to cuprous oxide. Treat 5 cc. of 0.5% dextrose solution with not more than 2 cc. of 5% NaOH. Copper sulphate solution is now added, drop by drop, until a very small amount of the hydroxide remains undissolved in the liquid. Warm the solution. A yellow hydrated oxide or red sub-oxide separates out even below the boiling temperature. If too little copper salt has been added, the test will be yellowish brown in color, as in Moore's test. An excess of copper salt should also be avoided, since upon boiling the excess of hydroxide is converted into a dark brown hydrate, which interferes with the test. To prevent these difficulties, the so-called Fehling's solution is employed.

34.—Fehling's Test—Qualitative

Mix 10 cc. of the Fehling's copper solution with 10 cc. of the Fehling's alkaline solution in a test tube, and heat to boiling. The solution should remain clear. Divide the solution into three parts. To one portion add a few drops of glucose solution. Note the results. Continue the addition of the glucose, drop by drop, boiling between the addition of each drop, until no more blue color remains. Pour the contents of the test tube into an evaporating dish, and allow it to remain in the air for some time. What is the effect of atmospheric oxygen upon the solution?

To the second portion add a few drops of glucose solution, but without heating.

To the third portion add a few drops of diabetic urine, and heat.

35.—Quantitative Determination of Sugar—Fehling's Method.

Pipette out 10 cc. of the Fehling's copper solution into a 250 cc. flask. Add 10 cc. of the Fehling's alkaline solution and dilute with 50 to 60 cc. of water. Heat the solution to boiling. Place the glucose solution in a burette and add the solution drop by drop, boiling after each addition to the Fehling's solution. Continue until no blue remains. To determine the absence of blue, the meniscus of the liquid should be examined against a light background. If a volume of less than 5 cc. of glucose is required, dilute the solution so that at least this amount shall be used, and repeat the test, this time running in nearly the required volume

at first. Toward the end point, add the solution drop by drop as before. Calculate the percentage of sugar in the solution, considering that the Fehling's solution has been reduced by 0.05 grams of glucose; that is to say, the volume necessary to completely precipitate all of the copper must contain this amount of dextrose. Report the percentage of sugar in the unknown specimens furnished, to the instructor before proceeding. Note: Fehling's solution is prepared in the following manner: Solution A (copper sulphate solution)—17.325 grams of purest obtainable copper sulphate are weighed out and dissolved in water, and made up to 500 cc.

(10 cc. equals 0.050 grams of glucose.)

Solution B (alkaline tartrate solution)—86 grams of Rochelle salts and 25-30 grams of potassium hydroxide are dissolved in water and made up to 500 cc.

These solutions must be preserved separately. Before using, 10 cc. of each of the solutions are mixed, diluted and boiled as described above. The tartrate holds the excess of cupric hydroxide in solution, and an excess of the reagent does not, therefore, interfere in the performance of the test.

36.—Benedict's Test—Qualitative

Several copper reduction tests for carbohydrates have been originated within the last twelve years, claiming superiority to the original Fehling's test. Part of this contention is based upon the fact that small amounts of sugar present in a fluid may be decomposed by the caustic alkalinity of the Fehling's solution. A second factor to be considered is that uric acid and creatinine present in urine have also a reducing action upon Fehling's solution.

The chief objections to the use of Fehling's solution from the practical standpoint is not due to any inherent fault of the method, but largely to the inability of the person conducting the estimation to determine readily the disappearance of the last trace of blue color, indicating unreduced copper.

The operator will be well repaid if he takes the trouble to master the required technic. It is briefly as follows: Find the clear hair line, representing surface reflection, which may always be observed at about 1/16 to 1/4 inch below the surface of the hot liquid (contained in a 250 cc. Florence flask), when lowered

slowly from a point just above to a point just below the level of the eye. A blue color indicates that more sugar solution should be added. When colorless or water clear, the end point is obtained, while a yellow or yellow green color indicates that an excess of sugar has been added.

Benedict's test was invented to banish all the faults of Fehling's method. It is said to be more suitable for urine work on account of not being reduced by uric acid or creatinin. Other advantages are recited in many text books.

QUALITATIVE TEST

Place 5 cc. of Benedict's solution (qualitative) in a test tube. Add 3 to 4 drops of 0.5% glucose solution. Boil vigorously for 1 to 2 minutes, then place in the rack to cool. The tube should contain a large bulk of red, yellow, or green precipitate, depending upon the concentration of the sugar.

Note—Benedict's Qualitative Solution.

Weigh out 173 grams of sodium citrate, add to a beaker containing 600 cc. of boiling water. Stir until dissolved. Now weigh out 90 grams of anhydrous sodium carbonate, and add to the citrate solution, little at a time and with stirring. If necessary filter through paper. Make the volume of these solutions up to 850 cc. when cold.

Weigh out 17.3 grams of crystalline copper sulphate. Dissolve in 100 cc. hot water, and when cold dilute to 150 cc.

Now pour *slowly* and with *stirring*, the copper sulphate solution into the carbonate-citrate solution. When mixed, the solution should remain clear indefinitely.

37.—QUANTITATIVE DETERMINATION OF SUGAR — Benedict's Method.

Pipette 25 cc. of Benedict's quantitative solution into a 250 cc. Florence flask. Add 8-10 grams of solid sodium carbonate, and heat the flask to boiling. The carbonate should be dissolved in a minute or two. The fluid in the flask *must be kept gently boiling during the entire addition of the sugar solution.* With the flask gently boiling run in the sugar solution (or urine) from a burette, *slowly,* until the flask is opaque, from the formation of a white precipitate. The blue color will now be rapidly disappearing. Now add the sugar solution, cautiously, a few drops at a time until the last trace of blue has disappeared. If the

volume of sugar solution required is *less* than 10 cc. dilute it with water so that from 10 to 15 cc. will be required. Repeat the determination with the diluted solution thereby obtaining greater accuracy.

25 cc. of Benedict's solution equals 0.05 gm. glucose.
25 cc. of Benedict's solution equals 0.053 gm. fructose.
25 cc. of Benedict's solution equals 0.074 gm. maltose.
25 cc. of Benedict's solution equals 0.0676 gm. lactose.

Note: Benedict's quantitative solution is prepared as follows:

Weigh out and dissolve in 500 cc. of hot water, 200 grams of sodium citrate, 100 grams of crystalline sodium carbonate and 125 grams of sodium or potassium sulphocyanide. When dissolved, allow to cool, then transfer to a 1,000 cc. volumetric flask.

Weigh out exactly 18.0000 grams of purest obtainable copper sulphate crystals. Dissolve with the aid of heat in 100 cc. of distilled water.

When both solutions are cold, pour the copper sulphate solution without loss into the alkaline citrate solution. The volumetric flask should be almost continuously shaken during the addition of the copper sulphate solution and but little of the latter should be added at a time.

Now add 5 cc. of a 5% solution of potassium ferrocyanide to the mixture in the flask, then fill to the 1000 cc. mark by thoroughly rinsing the beakers used for dissolving the copper sulphate solution, and the alkaline citrate solution into the flask. Thoroughly mix the solution.

Benedict's solution possesses the advantages of having a white end-point. The reduced copper, instead of settling out as a reddish cuprous oxide, as in Fehling's solution, unites with the thiocyanate, forming the colorless precipitate. The addition of the small amount of potassium ferrocyanide is to prevent decomposition of the cuprous oxide.

Each student is required to obtain checks with a 0.5% glucose solution, and to do and report one unknown.

Formation of Osazones—Reaction with a Phenyl-hydrazine.

All reducing sugars react with either phenyl-hydrazine or a substituted phenyl-hydrazine to form crystalline compounds.

Certain sugars are capable of forming easily identifiable compounds with but one molecule of phenyl-hydrazine. These compounds are called *hydrazones.* If an excess of phenyl-hydrazine is present, then three molecules will react with each molecule of reducing sugar. The product in this case is an *osazone.* This reaction is also given with certain substituted phenyl-hydrazines.

It is important to remember that those monosaccharrides sharing a common enolic form give the *same identical osazone.* As sugars are identified by the shape of crystal and especially by the melting point of *the osazone,* the ability to form enols must not be overlooked.

To obtain satisfactory results, if the base phenyl-hydrazine is used, it must be colorless, or at most, of a very light yellow. Dark-colored samples are contaminated by an oxidation product, and require distillation in vacuo (15 mm.), followed by further purification. In this laboratory we find it more convenient to use phenyl-hydrazine hydrochloride. The commercial samples are invariably impure and should be purified by recrystallization from hot alcohol. The material should be filtered upon a Buchner funnel as soon as dissolved. The filtrate cooled, and the separated platelets filtered on a second Buchner, preferably in very subdued light. The mother liquor should be sucked out as thoroughly as possible, then the platelets removed and air dried in *a dark place.* When dry the platelets are glistening and pearly white. Keep in a stoppered brown glass bottle.

38.—Preparation of Phenylglucosazone

Place in a test tube 0.2 gram purified phenyl-hydrazine hydrochloride, and 0.3 gram crystalline sodium acetate. Add 0.1 gram glucose (or 10 cc. of a 1% solution) and water to a total volume of 20 cc.

Place the tube in a hot water bath and maintain just at the boiling point for 1½ hours, remove the flame and allow the tube to cool slowly *in the water bath.* Note and record in this and subsequent work the appearance of crystals if in the hot solution or only upon cooling. Remove a few of the crystals to a microscope slide, examine and sketch.

If time permits, the crystals of monosaccharides may be filtered off and recrystallized from hot alcohol or pyridine, dried, and their melting point determined.

Osazones of disaccharides may be purified by recrystallization from hot water.

The phenylosazones of the common sugars, have the following melting points:

Phenylosazone of d-Arabinose, 160° C.

Phenylosazone of d-Glucose, 208° C.

Phenylosazone of d-Mannose, 208° C.

Phenylosazone of d-Galactose, 193° C.

Phenylosazone of Maltose, 206° C.

Phenylosazone of Lactose, 200° C.

In determining melting points, the heating should be rapid, to the point at which melting is first observed. Immediately withdraw the heat and note temperature and completeness of fusion, with or without decomposition (gas development).

39.—Phenylosazone from Urine

Under certain conditions it is of the utmost importance to be able to distinguish between a pentose (usually d-l-arabinose), glucose, or lactose in urine. Pentoses are found occasionally in urines, usually of people addicted to the morphine habit; glucose in urines of diabetics and others committing a dietary indiscretion; lactose, often in the urine of women during certain stages of pregnancy, or after child birth. *Urines to be tested must be free from albumen.*

The technic as outlined by Emil Fischer is given below:

To a tube containing 10 cc. of urine add 0.2 gram purified phenylhydrazine hydrochloride. Add 0.4 gram crystalline sodium acetate. Heat for 1½ hours in a gently boiling water bath.

The amorphous precipitate is the crude and impure osazone. Filter this off; dissolve in a little hot alcohol (50%); filter again if necessary; add a little water, then as the alcohol evaporates, characteristic yellow crystals appear. Examine under the microscope, and after drying, take the melting point.
ides will be referred to again in proper order.

Note: The student is expected to make osazones of glucose, arabinose, maltose, and lactose at this time. He is expected to practice the technic required for identifying glucose or lactose when 0.1 gram is added to 10 cc. of urine. The disaccha-

ides will be referred to again in proper order.

40.—Nylander's Test—Reduction of a bismuth salt.

To 10 cc. of 0.5% glucose solution add 2 cc. of Nylander's reagent. Place the tube in a boiling water bath for 5 minutes. A black precipitate of metallic bismuth separates out. Note: Nylander's reagent is prepared by dissolving 50 grams of Rochelle salts together with 20 grams of bismuth subnitrate in 1 liter of 8% sodium hydroxide.

This test is not effected by urates or creatinine, but is by albumin. It is also useful in distinguishing homogentisic acid, found in alkaptonuria from glucose.

The student should repeat this test with arabinose, and also with the disaccharides.

41.—Barfoed's Test—Reduction of Copper Acetate in Dilute Acetic Acid Solutions.

Put 5 cc. of Barfoed's reagent in a test tube. Add 1 cc. of 0.5% glucose and heat the tube from 3 to 4 minutes in the boiling water bath. Examine for the presence of a precipitate. Repeat this test with the disaccharides. Is this test specific for a monosaccharide?

Note: To prepare Barfoed's reagent, dissolve 45 grams of neutral copper acetate crystals in 900 cc. of distilled water. Filter if not clear. Add 1.5 cc. of 50% acetic acid, and dilute to 1 liter.

42.—Fermentation by Brewer's Yeast

Fill a small test tube full of 0.5% glucose solution. Add ⅛ to ¼ cake of Fleishmann's compressed yeast. Rub the yeast to a fine suspension by means of a glass rod, then transfer to a fermentation bulb, stopper and invert, or use a Durham Fermentation tube, plugged with cotton. Place in a suitable beaker. Allow the apparatus to incubate in the warm room until the next period, then examine for evidence of fermentation. Note the volume of gas formed, and test a clear filtered portion of the remaining liquid for alcohol by means of the iodoform test.

Run simultaneously a control containing only yeast and distilled water. Note: To perform the iodoform test, aerate 5-10 cc. filtered fermentation mixture, which is slightly warmed by being placed in a water bath, into 3-4 cc. of a mixture of iodine solution, just decolorized by the addition of 40% NaOH. For further details consult the instructor.

This test should be applied to all monosaccharides (including a pentose) and also to disaccharides as indicated below. Note: To avoid confusion, it should be remembered that brewers' yeast contains an enzyme capable of hydrolysing certain disaccharides to monosaccharides. These then ferment. Pure yeast ferments only four monosaccharides. Which are they? Prove it by experiment. Conjugated glucuronic acids (occurring in urine after taking menthol, turpentine, etc.) reduce copper and bismuth solutions, but do not ferment.

43.—Detection of a Pentose—Bial's Orcin Test.

Place 5 cc. of Bial's reagent in a test tube. Heat just to the boiling point, then remove from flame. Now add *drop* by *drop*, the pentose solution. Not over 25 drops should be added. If pentose is present, the solution turns a beautiful green (Hammarsten) or violet blue (Abderhalden).

This test is not influenced by glucuronic acids in urine because the solution is not boiled. Abderhalden claims that a spectroscopic examination of the fluid is unnecessary. Note: Bial's Reagent—Dissolve 1 gram of orcin in 500 cc. of 30% hydrochloric acid. Add 25 drops of a 62.9% (crystalline) ferric chloride solution (German "liquor ferri").

44.—Pentose—Other Reactions

Repeat test 30, 31, 34, 36, 38, 40, and 41, with a 0.5% arabinose solution.

45.—Detection of Fructose—Seliwanoff's Test

Place 5 cc. of Seliwanoff's reagent in a test tube. Add a few drops of a 1% fructose solution, then heat the solution just to boiling. A red-colored fluid *and a red precipitate* indicates fructose. The precipitate dissolves upon the addition of alcohol.

Note: Seliwanoff's Reagent: Dissolve 0.05 gram of resorcin in 100 cc. of concentrated hydrochloric acid. To this solution add 100 cc. of distilled water.

If glucose is present instead of fructose, the solution may color if boiled, but no precipitate is usually obtained.

46.—Fructose—Other Reactions

Repeat tests 30, 31, 34, 36, 38, 40, 41, 42, and 43 with a 1% fructose solution.

POLARIMETRY

Optical Activity

Certain substances, among which are various solids, liquids, vapors and solutions, have the ability, when placed in the path of polarized light, of rotating the plane of polarization. These substances are said to be optically active. The rotation varies both in amount and in sense of rotation. If the rotation, relative to the direction of propagation, is to the right, the substance is said to be dextro-rotatory and a plus mark is placed in front of the figure indicating the amount in angular degrees. If to the left, the substance is laevo-rotatory and is indicated by a minus sign preceding the angular degrees of rotation. Substances not rotating plane-polarized light are said to be inactive.

Plane-polarized light (vibrations proceeding in a single plane) is readily obtained by the so-called "Nicol" prism. This consists of a prism of Iceland spar, the natural angles of which are ground down until 68 and 90 degrees, respectively, are obtained. The rhombohedron is then cut into two 90° angles. After polishing, the wedges are cemented together by Canada balsam, thus restoring the rhombohedron.

Mono-chromatic light is admitted through the end of the prism. It is refracted into two rays. The extraordinary ray is refracted but little and consequently emerges in a single plane from the opposite face of the prism. The ordinary ray is refracted to such an extent that upon reaching the film of Canada balsam cementing the two halves of the prism together, it is reflected to the side of the prism and completely absorbed by the black varnish covering its wall.

Monochromatic light must be used with the polariscope. Such light is obtained by vaporizing a sodium salt in a Bunsen flame, and has a wave length of 0.00005893 cm. This is the average for the values of the two "D" lines. A mercury vapor lamp is often used, because the wave length of the green light obtained is shorter than that of the sodium light. Its value is 0.0000546074 cm. With a mercury vapor lamp, the observed rotation is greater than with sodium light. This is due to the shorter wave length of the former.

The polarimeter or polariscope is an instrument designed

to measure the rotatory polarization of a substance. For general use, the rotation is measured in degrees of arc.

The design of each polarimeter should be explained by the laboratory instructor. In general, the simplest polarimeter consists of two Nicol prisms—the first (near the source of light) is called the "polarizer" and plane-polarizes the entering light. The second Nicol prism is mounted in a headpiece capable of rotation around the optical axis, and is provided with a graduated scale and vernier. A space is left for placing the fluid to be examined in the path of light between the prisms. Adequate auxiliary lenses and other optical parts to increase the sensitiveness of the apparatus are provided. (Consult any book on physics.)

The field of view of a simple polariscope consisting of but two prisms is dark when the prisms are crossed. This is taken as the zero point of the apparatus. If an optically active substance is now placed between the prisms, the beam of light is rotated and the field appears brighter. By rotating the analyzer until the light is again extinguished, one can measure the amount of rotation due to the optically active substance.

To increase the sensitivity of the apparatus, the field of observation is divided into halves (Laurent half-shadow). A plate, one-half of which is glass, while the other half is quartz, cut parallel to its axis, is placed just back of the polarizer. The thickness of the quartz plate is such as to retard the sodium light exactly one-half wave length. This causes the two halves of the field to appear unequally illuminated except when the principal plane of the analyzing Nicol is parallel to the axis of the quartz. At this point the two halves appear uniformly illuminated. By changing the position of the polarizer with respect to the quartz plate the sensitiveness of the apparatus can be varied. The zero point is that position of uniform field illumination; the dark change being taken on account of greater sensitiveness.

Triple shade polariscopes have auxiliary Nicol prisms. These increase the sensitiveness and ease with which the apparatus can be read. Other types of instruments using quartz wedges are described in any book on the subject.

Many organic substances, like sugars, proteins, etc., contain within their structure one or more asymetric carbon atoms, i. e.,

a carbon atom to which is attached no two similar elements or groups of elements. These asymetric carbon atoms have the power of rotating the plane of polarized light to a greater or less extent, depending upon the number of molecules through which the polarized light passes.

The *specific rotation* of a substance is the rotation expressed in degrees which is given by 1 gram of a pure substance dissolved in 1 cc. of water, and viewed through a tube one decimeter in length. Under these conditions, we have unity of volume, unity of mass, and a unit length, therefore, unity of concentration.

The specific rotation $(\alpha)_D^{20}$ may be calculated by means of the following formula:

$$(\alpha)_D^{20} = \frac{a}{c \times l}$$

in which:

D is sodium light.

a is observed rotation in degrees.

c is grams of substance dissolved in 1 cc. of fluid.

l is length of the tube in decimeters.

After determining the specific rotation of any optically active substance we may determine either the concentration or the per-cent of the substance in an unknown solution. To determine the percentage, use the following formula:

$$\text{Per cent} = \frac{a \times 100}{(\alpha)_D^{20} \times l}$$

The following specific rotations are taken from Cole:

	$(\alpha)_D^{20}$	$(\alpha)_{Hg}^{20}$
Glucose	+52.5	+62.
Lactose hydrate	+52.4	+61.9
Lactose anhydride	+55.2	+65.2

Maltose	+138.0	+163.0
Sucrose	+66.5	+78.5
Fructose	—93.8	—110.8
d-galactose	+81.0	+95.7
l-xylose	+19.0	+22.4
Invert sugar	—20.6	—24.6

47.—Determination of Specific Rotation of Cane Sugar

Weigh out exactly 10 grams of pure cane sugar and dissolve in water. Transfer to a 100 cc. volumetric flask, and make to volume at 20° C. Polarize immediately, then set aside until the next laboratory period and polarize again. The second reading multiplied by 10 should give a figure closely agreeing with that given in the table if a decimeter tube is used

After determining the specific rotation, pipette 50 cc. of the sugar solution into a beaker—add 0.5 cc. of acid and boil for fifteen minutes, supplying the water lost by evaporation, and read again. What is happening to the cane sugar? What is the meaning of the term "invert sugar"?

Determine the percentage in two unknowns and report to the instructor.

Add about 1 gram of glucose to 100 cc. of urine. Determine the percentage of glucose by means of the polariscope and compare the result with that obtained by Fehling's or Benedict's method.

Disaccharides.

Disaccharides should be looked upon as glucosides of monosaccharides. Maltose is glucose-α-glucoside. Lactose is glucose-β-galactoside. If the reducing group of one component is free, then the disaccharides will exhibit corresponding reactions (lactose and maltose). If both active groups are involved in the condensation, the resultant molecule is relatively inert (sucrose).

48.—Maltose—Preparation from Starch

Mix 25 grams of starch with 30 cc. of cold water until a smooth paste is obtained. Pour this slowly and with stirring into 350 cc. of boiling water in a 500 cc. beaker. Continue the boiling for one or two minutes until a smooth paste is obtained. Allow to cool to 60°-70° C. Place in a water bath at this temperature,

and stir in a teaspoonful of malt. Maintain at this temperature until the mixture becomes thin and watery. Remove from the water bath, carefully drying the outside of the beaker, and heat to boiling again . What is the object of this heating? Boil for one or two minutes. Cool to 60° C. and add another teaspoonful of malt and keep at this temperature by means of the water bath until the sample no longer gives any color with iodine solution. Remove from the water bath, dry the beaker, boil, filter, and taste. Allow to cool. Pipette out 10 cc. of the solution into a 100 cc. volumetric flask. Add water up to the mark. Make a quantitative determination for sugar. Remove 50 cc. of the solution just titrated, add 5 drops of concentrated hydrochloric acid and boil for fifteen minutes. Cool. Neutralize the acid by means of sodium hydroxide (using litmus paper) and make up to 50 cc. volume. Determine once more the reducing power of the solution and compare with that of the above. Explain. What is the reducing power of maltose compared with that of glucose?

If fresh malt is not available, this experiment may be carried out by adding 0.5 gram of Taka-Diastase (Park-Davis & Co.) in place of the spoonful of malt.

49.—Maltose—Sugar Reactions

Reactions numbered 31, 34, 38, 40, 41, 42, and 43 are to be performed with the maltose solution just prepared, and compared with those obtained when using other sugars.

50.—Lactose—Preparation from Milk

Dilute 300 cc. of skimmed milk with 500 cc. of water. Add acetic acid cautiously—*drop by drop*—with constant stirring until the precipitation of casein is evidenced by curd formaton. Now cautiously continue the addition of a 25% acetic acid solution until no further precipitate is formed when a drop of dilute acid is added to a filtered test portion. The floculent precipitate settles readily. An excess of acetic acid is avoided, to prevent redissolving the casein. Filter the solution, catching the filtrate in a large beaker. Save the precipitate and place in the receptacle provided.

Heat the filtrate to boiling. Coagulation of the albumin occurs, which, when complete, is removed from the solution by filtration through a plaited filter paper. Evaporate the filtrate

upon the steam bath until a thin syrup is obtained. The solution is now allowed to evaporate spontaneously until crystals of lactose separate out. Do not carry evaporation so far that crystals cannot be separated from the mother liquor! Separate the crystals by filtration (through cloth), then if desired (although not necessary), purify by recrystallization from water.

Rub a few crystals between the fingers. Why is milk sugar sometimes called "sand sugar"? Is milk sugar sweet? Taste it. With the remainder of the preparation, make a solution of approximately 1 per cent.

51.—Lactose—Sugar Reactions

Reactions numbered 31, 34, 38, 40, 41, 42, and 43 are to be performed with the solution prepared in the last experiment, and compared with those obtained when using other sugars.

52.—Lactose—Mucic Acid Test

Measure 100 cc. of 1 per cent solution of lactose into a suitable evaporating dish. Add 20 cc. of concentrated nitric acid, and place on the steam bath. Note the brown fumes. The lactose is oxidized to mucic acid, $COOH\text{-}(CHOH)_4\text{-}COOH$.

After the reaction is complete, continue the evaporation until a final volume of 20 cc. remain. Transfer the solution to a small beaker, rinsing the evaporating dish with a small quantity of distilled water. If the beaker is put in a cool place for 24 hours, white crystals of mucic acid should appear. Add a little water to the beaker, then separate the crystals from the mother liquor by filtration. Wash and examine under the microscope. The crystals should be sharply pointed prisms with oblique angles. Melting point, 213°-215° C.

Note—The mucic acid test is not dependable enough for use when distinguishing between lactose and glucose in urine.

53.—Lactose in Urine—Cole's Test

To 1 gram of good charcoal (Merck's purified blood charcoal, washed with hot hydrochloric acid until free from phosphates, then washed free from chlorides) in a large test tube, add 25 cc. of the suspected urine. The contents of the tube should be mixed by shaking, boiled for a few seconds, cooled thoroughly under the tap, and vigorously shaken for 10 minutes. The material is filtered by suction, and the charcoal drained as thoroughly as possible. Transfer the charcoal to a small evapor-

ating dish, freeing it from the filter paper, then add 10 cc. of distilled water and 1 cc. of glacial acetic acid. Stir the charcoal with a glass rod and then transfer the mixture to a large test tube. Heat to boiling for about 10 seconds, filter while hot, collecting the filtrate in a test tube containuing 0.2 gram of purified phenylhydrazine hydrochloride and 0.3 gram of sodium acetate.

Proceed to form the osazone as directed in experiment 38. Identify the crystals. If necessary, recrystallize the osazone by dissolving the precipitate in 4 to 5 cc. of hot water, filtering several times through a small paper, then cool. The solution must be heated after each filtration to prevent the crystals from forming in the paper during filtration.

Note: The lactose is absorbed by the blood charcoal. Each charcoal should be carefully tested with solutions of known lactose content before being considered suitable for the above test.

Mathews distinguishes between lactose and glucose by their respective fermentability with ordinary yeast. He first estimates the total reducing power of a solution or urine by Benedict's method. Then one-half cake of yeast is shaken in a large tube with 20 cc. of urine or solution until uniformly mixed. If glucose is present, it will be completely fermented in 50 minutes, providing its concentration is not over 6 per cent, if the tube is placed in a beaker of water maintained at 42° C., and the yeast kept in suspension by inverting the tube at frequent intervals. The unfermented lactose is then determined in the solution after filtration, by Benedict's method. Subtracting this value from that previously obtained gives the percentage of glucose. The lactose may be still further identified by preparation of its osazone.

54.—Sucrose—(Cane Sugar)—Sugar Reactions

Reactions numbered 31, 34, 38, 40, 41, 42, and 43 are to be performed with a 1% sucrose solution and contrasted with those obtained when using other sugars. To what is the difference in reactivity due? Review experiment 47 at this time.

55.—Sucrose—Hydrolysis or "Inversion"

To 3 cc. of a 1% sucrose solution add 2 or 3 drops of concentrated hydrochloric acid. Boil for a few seconds. Cool under the tap. To the solution add ½ cc. of 20% copper sulphate, 3 drops of glycerol, and 3 cc. of 20% sodium hydroxide.

Boil. Explain the reduction. What is the glycerol added for?

56.—Problem

Identify the sugar or sugars present in two unknowns. Report your results to the instructor, stating reasons for your opinion as to the identity of the sugars.

Polysaccharides

Polysaccharides are formed by condensation from monosaccharide molecules (water being eliminated).

Two groups are commonly encountered, namely:

1. The Pentosans $(C_5H_8O_4)n$ which, upon hydrolysis, yield the pentoses. Example of this group are various gums, as gum arabic, and cherry tree gum.

2. The Hexosans $(C_6H_{10}O_5)n$ which, upon hydrolysis, yield glucose. Starch, cellulose, dextrin and glycogen are representatives of this class.

57.—Starch—Microscopic Appearance and Solubility

Sketch the granules of the following starches: Potato, corn, wheat, and rice. Note their shape, the concentric markings, if any, and the hilum. Add a drop of dilute iodine solution to one of the varieties and re-examine. Record the result.

Solubility of starch: Add a little powdered starch to cold water. Filter, and add to the filtrate a drop of iodine solution. What are your conclusions?

Gelatinization (Cole): Shake some dry starch with a little 20% sodium carbonate solution. No change is effected. Repeat, substituting 20% sodium hydroxide. Note the appearance; add a few drops of iodine solution. Why do you not obtain a color? Add glacial acetic acid. What happens? Why?

58.—Preparation of a Starch Paste

Note: Starch consists of two substances—

(a) Amylopectin or starch cellulose, forming about 60% of the grain, serves to enclose it. It is insoluble in cold water, swells (imbibition) without dissolving in hot water. A similar gelatinization occurs when starch is treated with cold sodium hydroxide.

While acids hydrolyse amylopectin to glucose, amylases (diastases) convert it into maltose and a dextrin which is very resistant to further hydrolysis. The opalescence of a starch paste is due to amylopectin.

(b) Amylose, or granulose, the material within the grain, is protected by the external layer of amylopectin. Amylose is soluble in cold water, is completely hydrolysed to maltose by amylases. It gives the characteristic blue color when treated with iodine solution.

Starch Paste: Rub 2 grams of starch to a thin paste with a little water and pour the mixture slowly with constant stirring into 200 cc. of boiling water. Use the paste so obtained in the following experiments.

59.—Starch—Properties and Precipitation Reactions

(a) To some of the starch paste in a test tube add a drop of dilute iodine solution. Note the result. Place the tube in a beaker of water and heat gradually. What change takes place? Allow the solution to cool and observe the result.

(b) Add a few drops of a solution of tannic acid to a few cc. of the starch solution. A yellow precipitate, soluble upon the application of heat, is obtained.

(c) To a few cubic centimeters of starch paste in the test tube, add alcohol until a definite precipitation occurs.

(d) To a few cubic centimeters of cold starch paste, add an equal volume of saturated ammonium sulphate solution. Shake the tube, allow to stand for 5 minutes, then filter. Test the precipitate and filtrate with dilute iodine solution. Is the precipitation complete?

60.—Starch—Hydrolysis by Acid and Enzyme

(a) Test a portion of starch solution with Fehling's solution and record the result.

(b) To 25 cc. of starch solution in a flask add 4 or 5 drops of concentrated hydrochloric acid, or a few drops of dilute sulphuric acid, and boil for fifteen minutes. What change do you observe in the appearance of the starch solution? Neutralize some of this solution with sodium hydroxide solution, and apply Fehling's test. Report the result. (If no reduction takes place, continue the boiling for another fifteen minutes and repeat the test.) What sugar is formed?

(c) To 5 cc. of starch solution in a test tube, add 3 cc. of saliva and place in a water bath at 40° C. After twenty minutes, test for reducing sugars by Fehling's and by Barfoed's tests. What variety of sugar do you find present?

61.—Dextrin—from Starch

The two following procedures, together with the note, are taken from Cole's Physiological Chemistry.

(a) Erythro-dextrin: Measure 2 cc. of starch paste into a test tube, add 6 drops of concentrated hydrochloric acid from a pipette, then heat to boiling and maintain the boiling for *one minute by the watch.* Cool thoroughly under the tap. Add first one drop, and then another drop of dilute iodine solution. A red or violet color is produced, indicating the conversion of starch into erythro-dextrin by acid hydrolysis.

(b) Reduction test: (Probably due to the glucose formed simultaneously with erythro-dextrin.) Boil 2 cc. of starch paste with 6 drops of concentrated hydrochloric acid as before. Cool. Add 3 drops of glycerol and 8 drops of 20% copper sulphate. Add 20% sodium hydroxide until a grey precipitate is produced. Now add another 2 cc. of the sodium hydroxide and boil for a minute. A slight reduction is obtained.

Note: If 12 drops of hydrochloric acid be added and the mixture boiled for 1 minute, it will generally be found that only a yellow color is produced with iodine and that the amount of glucose formed is not sufficient to reduce 9 drops of copper sulphate. At this stage a considerable proportion of the carbohydrate is in the form of achroo-dextrin. It is important to note that complete hydrolysis of starch by acids is relatively slow compared to that of sucrose and the other disaccharides.

62.—Dextrin—Properties

Using a 1% aqueous solution of dextrin, perform the following tests: No. 59 b, c, d, and No. 60 a, b, c. Contrast in each case with starch.

Glycogen

Glycogen is so resistant to the action of boiling alkali that this reagent may be used for its preparation from muscle or liver tissue. Pure glycogen is colorless, dissolves in water to an opalescent solution. It may be precipitated from its solution by addition of an equal volume of 95% alcohol or by completely saturating its solution with (solid) ammonium sulphate.

It is relatively inactive, as indicated by the absence of reducing power. It gives a characteristic port-wine color with dilute

iodine solution. Yeast cannot ferment glycogen, while diastatic enzymes, except those occurring in the liver, hydrolyse is to maltose. The glycogenase of the liver hydrolyses it to glucose. Its solution is dextrotatory.

63.—Glycogen—Preparation from Animal Tissue

Oysters, scollops, or the liver of a recently killed animal serves well as material for this preparation. If dogs are available, and especially if needed for other work, their livers may be used most satisfactorily, especially if the animals are fed upon boiled potatoes about six hours before being killed.

Each student should obtain 30 grams of fresh material, and immediately, after grinding as finely as possible, drop it into a flask containing 200 cc. of boiling water, which is slightly acidified with acetic acid. As soon as the protein has coagulated, the material is filtered off, using a plug of cotton in the funnel, and catching the filtrate in a flask. Place the residue in a mortar, and after adding coarse sand, grind thoroughly. Return the material to the filtrate, and boil for a few minutes. Filter hot through a cloth or plug of cotton in a funnel, collecting the opalescent fluid in a flask. Press the fluid out of the residue as thoroughly as possible, and rinse with a little water.

Evaporate the filtrate to a volume of 75 to 100 cc. Add to the filtrate alternately a few drops of hydrochloric acid and potassio-mercuric iodide until a precipitate of proteins ceases to form. This may be determined most conveniently by filtering off a small portion of the liquid from time to time, and adding to the clear filtrate a drop of hydrochloric acid and a drop of potassio-mercuric iodide. When the precipitation of proteins is complete, filter and to the milky filtrate add double its volume of alcohol; the glycogen will precipitate as a white powder. Filter this off, wash with 66% alcohol and dissolve in water. With this solution perform the following tests.

64.—Glycogen: Properties—Reactions—Digestions—Hydrolysis

(a) Add a drop of iodine solution to a portion of the glycogen solution. The liquid will assume a dark red color. This color disappears, with the exception of the color due to iodine, upon gentle heating, and reappears upon cooling. (Compare with dextrin.)

(b) Test with Fehling's solution and report the result.

(c) Add basic lead acetate to some of the solution. (Compare with dextrin.)

(d) Add some saliva to portions of the glycogen solution and heat the mixture for twenty minutes in a water bath at 40° C. Remove and divide into two portions. Test one with iodine solution for glycogen and the other with Fehling's solution for sugar. Report the result.

(e) Boil a portion of the glycogen solution with a few drops of dilute sulphuric acid. Neutralize with sodium hydroxide and test with Fehling's solution. Report the result.

COLLOIDS

Graham in 1861 showed that potassium hydroxide, potassium sulphate, magnesium sulphate, sugar and alcohol diffuse more rapidly in water than hydrous silicic acid, hydrous alumina, starch, dextrin, gums, albumin, and gelatin. The latter substances usually occur in non-crystalline or gelatinous condition, and therefore, as they resembled glue in this particular, were called colloids by him. Substances of the first group, crystallize readily in a definite way, therefore those substances capable of ready diffusion usually resemble them in their ability to crystallize and were grouped together as a class called crystalloids. Today the distinction between colloids and crystalloids indicated above has been largely dropped. We now speak of a *colloidal state* instead of a colloidal substance. This indicates that any substance in a sufficiently fine state of subdivision or "dispersion," as it is called, may constitute one phase of a colloidal system. The subdivided phase is called the internal or dispersed phase; while the enveloping phase is called the external or continuous phase.

The student should consult the following texts for further information upon this subject: Holmes—Laboratory Manual of Colloidal Chemistry; Bancroft—Applied Colloidal Chemistry; and Loeb—Proteins and the Theory of Colloidal Behavior.

65.—Preparation of a Mastic Suspension

Prepare a saturated alcoholic solution of gum mastic. To a beaker containing 100 cc. of distilled water add 1 cc. of the mastic solution, *drop by drop*, with *stirring*. Filter off the coursely suspended particles. The suspension is electro-negative in character. To demonstrate the effect of electric charges of opposite as well as like nature, perform the following experiment:

Place 10 cc. of mastic suspension in each of four test tubes. Number them 1, 2, 3, and 4.

To 1 add 5 cc. N/10 HCl.

To 2 add 5 cc. N/10 NaOH.

To 3 add 5 cc. 6% NaCl.

To 4 add 5 cc. 10% sugar solution. +

1. Hydrochloric acid is abundant in positive (H) ions.

These have what effect upon the negatively charged mastic solution?

2. Sodium hydroxide is characterized by the negative ($\overline{OH}$) ions. Note the stabilizing effect of the ion with like charge. Now add a 6% sodium chloride solution to this solution. Note the quantity added and compare with the result obtained in tube "3."

3. Sodium chloride dissociates into sodium and chlorine ions. Which ion is responsible for the effect observed?

4. Sugar is a non-electrolyte. What is the nature of the precipitations observed above?

66.—Arsenic Sulphide Suspension

If hydrogen sulphide be passed into a solution of arsenious oxide, dissolved by the aid of sodium carbonate, a beautiful orange-colored solution of colloidal arsenic sulphide is obtained. This is also an electro-negative suspensoid.

With a solution of colloidal arsenic sulphide repeat the experiments given under No. 65.

67.—Colloidal Iron—"Dialysed Iron"

If a solution of ferric chloride be dialysed against distilled water, a slow decomposition occurs whereby a part of the chloride is changed over to ferric hydroxide. This mixture of ferric chloride and ferric hydroxide is known as "dialysed iron." It is electro-positive in nature.

Repeat experiment No. 65 using dialysed iron. Add the sodium chloride to tube No. 1, instead of tube No. 2 as directed. Explain fully the results obtained.

68.—Emulsion Colloids

Many proteins, gums, starches, etc., go into a colloidal state in solution. Only those properties exhibited by gelatin and casein can be considered here. The student should carefully consider the presence or absence of an electric charge, as well as its character in all cases where emulsoids are encountered. See Loeb—Proteins and the Theory of Colloidal Behavior.

69.—Geletin—Swelling or "Imbibition"

Place 5 grams of gelatin in a beaker and just cover with

water. Note what happens upon standing. Explain. Save the material for the next experiment.

70.—Reversibility of the "Sol" and "Gel" State

To the gelatin in the beaker in experiment No. 68, add just enough water to get it into solution when heated in a boiling water bath. This represents the sol state.

Remove the gelatin from the water bath and allow the beaker to cool. What do you observe? What is the relation of gelatin to water in the gel phase? Explain. Save the material.

71.—Reversibile and Irreversible Colloids

To the gelatin solution used in experiment No. 70, add sufficient warm water to make a total volume of 100 cc. Use when required in experiments below.

(a) Measure 10 cc. of this solution into an evaporating dish and place on the steam bath. Evaporate to dryness. Add water and determine whether the gelatin returns to its former state of solution. (Reversible Colloid.)

(b) Measure 10 cc. of arsenic sulphide solution into an evaporating dish and add water and compare the solubility of the residue with that of the gelatin. (Irreversibility.)

72.—Salting out of Emulsoid

To 25 cc. of a 2% solution of egg albumin, add solid ammonium sulphate until the solution is completely saturated. What happens? Filter off the precipitate. Add distilled water. Is it possible to redissolve the albumin? What is the difference between the precipitation here and that occurring in tube No. 3, experiment No. 65?

Iso-Electric Point of Emulsoids

Many proteins may be dissolved in dilute acid and in dilute alkali solutions. In the former they adsorb the hydrogen ion and become positively charged. In the latter the adsorption of hydroxyl ions imparts a negative charge to them. There is, however, a definite point of reaction for each colloid, attained by the adsorption of the proper amount of positive or negative ions to render the colloid neutral or without charge. The reaction of the solution at this time indicates what is known as the iso-electric point. This is the point (or reaction) at which precipitation occurs.

73.—Determination of Iso-Electric Point of Casein (Cole)

Into a 50 cc. measuring flask place 0.3 gm. of purified casein. Add about 25 cc. of distilled water, previously warmed to about 40° C. Add exactly 5 cc. of normal sodium hydroxide. Agitate until the casein dissolves, taking care to prevent frothing. Rapidly add 5 cc. of normal acetic acid, mix, cool, and make up to 50 cc. with distilled water. A faintly opalescent solution of casein in 0.1 N. sodium acetate is thus obtained.

Make up the following series of tubes, using clean, dry test tubes:

Tube number	1	2	3	4	5	6	7	8	9
Ccm. casein in 0.1 N. sodium acetate	1	1	1	1	1	1	1	1	1
Ccm. dist. water	8.38	7.75	8.75	8.50	8.00	7.00	5.00	1.00	7.40
Ccm. 0.01 N. acetic acid	0.62	1.25	...	...	...	...	...	...	...
Ccm. 0.1 N. acetic acid	...	...	0.25	0.50	1.00	2.00	4.00	8.00	...
Ccm. normal acetic acid	...	...	...	...	...	...	...	...	1.60

Place the casein solution in the tubes first, then the water, and mix. Now add the acetic acid to the first tube and *shake immediately*. Then add the acid to the second tube and shake this, and so on. Examine the tubes at intervals and record observations as below.

O=no change. +=opalescence. ×=precipitate.

Tube No.	1	2	3	4	5	6	7	8	9
On mixing									
After 10 minutes									
After 20 minutes									
H-ion conc'n									
pH value									

(Indicate intensity by one or more characters)

Calculate the approximate hydrogen ion concentration for each solution from the ratio of concentration of acetic acid and sodium acetate molecules as indicated in the following formula:

$$H = \frac{K \times (\text{acetic acid in mols. per liter})}{a \times (\text{sodium acetate in mols. per liter})}$$

H is Hydrogen ions in grams per liter.

K is dissociation constant of acetic acid.
is 1.85×10^{-5}

a is dissociation constant of sodium acetate.
is 0.87 for 0.01 N.

Example: Tube No. 1.

0.62 cc. acetic acid of $N \times 10^{-2}$ is diluted to 10 cc. Therefore the concentration is 0.62×10^{-3}. Put this value in the equation:

$$H = \frac{(1.85 \times 10^{-5}) \times (0.62 \times 10^{-3})}{0.87 \times 10^{-2}}$$

$$H = 1.32 \times 10^{-6}$$

To convert hydrogen ion concentration to the hydrogen ion exponent proceed as directed on page 21. Fill both values into the table above. Indicate clearly what the iso-electric point of casein is.

74.—The Influence of the Charge and Valence upon Precipitation (Cole)

Prepare a solution of casein in 0.1 N. sodium acetate as indicated in the last experiment.

To 2 cc. add 17.5 cc. of distilled water, and then 0.5 cc. of 0.1 N. acetic acid and mix quickly. A solution of casein is thus obtained, alkaline to the iso-electric point, and therefore carrying a negative charge. Divide the solution into four equal parts and place them into four clean tubes labelled -1, -2, -3, and -4. To another 2 cc. of the original solution of casein add 10 cc. of distilled water and 8 cc. of 0.1 N. acetic acid and mix quickly. An acid solution of casein is thus obtained. Divide into four parts, and place into four clean tubes labelled +1, +2, +3, and +4.

To the tubes marked 1 add 3 drops of normal KCl (7.45%).

To the tubes marked 2 add 1 drop of normal $BaCl_2$ (10.40 per cent).

To the tubes marked 3 add 1 drop of normal K_2SO_4 (8.7%).

Mix the contents of each tube and place them all in a water bath at 35°C. Examine and record results after 15 minutes, using the same characters as in the previous experiment.

Tube	Negative	Positive
1.		
2.		
3.		
4.		

Warm the tubes to 60° C. to still further increase the effect of the precipitating ion.

Study *carefully* the effect of charge on emulsoid, together with the charge and valence of precipitating ion. Distinguish clearly the difference between the results obtained here and those in experiment No. 72.

Write up your results fully, mentioning each tube.

75.—Mutual Precipitation Suspensoids of Opposite Charge

To 5 cc. of colloidal arsenic sulphide in a test tube add 5 cc. of dialysed iron solution. What happens? Explain.

76.—Protective Action of an Emulsoid for a Suspensoid

(a) To 5 cc. of colloidal arsenic sulphide solution add 10 drops of a 1% gelatin solution. Now add 6% sodium chloride solution, noting amount required to cause precipitation. Compare with experiment No. 66. Explain fully.

(b) Place 10 cc. of colloidal gold solution (prepared as for use in the Lange test) in each of two small evaporating dishes. Mark one G, and to this add 1 cc. of 1% gelatin. Evapo-

rate both solutions to dryness. Notice any difference in color. The courser particles of gold have a blue color. Add 10 cc. of distilled water to each and try to re-dissolve. Can you get the original solution back in either case? Why? Explain fully.

77.—Decrease in Agglomeration of Colloidal Precipitate by Strongly Absorbed Substance

Place 10 cc. of milk in each of two small beakers. Label them 1 and 2. To beaker 1 add 25 cc. of water. To beaker 2 add 25 cc. of 20% cane sugar solution. Add 10% acetic acid drop by drop to each, stirring vigorously, until *first evidence of coagulation is noticed.* Record the amount in each case. Is it different? Now notice the relative degree of fineness of the curds. Allow each beaker to stand for 5 minutes, then re-examine. Do you notice any change?

PROTEINS

Mülder in 1839 signified the importance of the substance of which flesh and all living cellular material is composed, by applying to it the term protein (from the Greek meaning "pre-eminence" or "of first importance"). This material; either of animal or plant origin; when carefully freed from fats, carbohydrates, extractives, and mineral salts, has a fairly uniform composition. It always contains the four essential elements, carbon, nitrogen, hydrogen and oxygen, and often in addition, smaller amounts of sulphur and phosphorus. In certain proteins, iron, copper or iodine in small quantities may be present.

The uniformity of composition of protein, especially in respect to the four named elements is striking. For the purpose of illustration, the following figures give some idea of the general composition of protein material as a class: Carbon, 50-52%; hydrogen, about 7%; nitrogen, 15-16%; sulphur, 0-3%; phosphorus, 0-3%, and 23-25% oxygen.

The colloidal nature of many proteins when in solution is clearly indicated in the precipitation reactions given below. The student should clearly distinguish between the phenomena of coagulation, precipitation, and "salting out" of proteins from their solution. The latter term is used here to indicate an agglomeration of the protein material without adsorption, and is to be contrasted with precipitation.

Proteins are composed of amino acids, linked together from amino to carboxyl group. The proteins may be unlinked by the action of certain proteolytic enzymes, as well as by acid and alkali hydrolysis. Fragments of the protein molecule are formed, varying in size and nature. The completeness of disruption of the molecule by an enzyme depends upon three factors: the nature of the protein; the particular enzyme used; and finally upon the length of time through which it acts. The rate of decomposition of proteins by acids depends upon the strength of the latter, and the completeness upon the time of action. Other factors such as temperature also effect the rate of the hydrolysis.

The color reactions, with the exception of the biuret test, indicate either the presence of a specific amino acid in the

molecule, or of a very limited number of amino acids which possess certain structural resemblance. The student is cautioned not to report the presence of protein in his solutions upon the basis of a single color reaction.

The study of protein chemistry is much simplified if the protein classification is kept clearly in mind. For that purpose it is given below in abbreviated form:

Protein Classification According to the American Society of Biochemists

Three classes are recognized. Several subdivisions occur under each class.

I. *Simple Proteins.* Naturally occurring proteins. Yield only alpha amino acid or their derivatives upon hydrolysis.

Name	Solubility in				Coagulability		Example
	Water	Dil. Salt	Dil. Acid	Dil. Alkali	Alcohol	Heat	
Albumins	+	+				+	Egg Albumin
Globulin	—	+				+	Serum globulin, Edestin.
Glutelins	—	—	+	+		+	Glutenin
Prolamines	—				80%		Gliadin, Hordein, Zein.
Albuminoids	—	—	—	—			Elastin, Keratin, Collagen.
Histones	+		+	Insoluble in Ammonia		—	Histones of Bird's Corpuscle.
Protamines				Soluble in Ammonia		—	Sturine, Salmin, Clupein.

Note: Histones and protamines are basic in nature. Histones have less basic nitrogen than the protamines, and usually contain sulphur.

II. *Conjugated Proteins.*—Compounds of simple proteins and non-protein group. The non-protein group is usually acid in nature.

A. Chromoproteins or Hemoglobins: Compounds of protein and a pigment bearing molecule. Example: Hemoglobin, hemocyanin, etc.

B. Glycoproteins (glucoproteins): Compounds of protein and a group which contains a carbohydrate molecule. Example: mucin.

C. Phosphoproteins: Compounds of protein and a group containing phosphoric acid other than nucleic acid. Examples: Casein and vitellin.

D. Nucleoproteins: Compounds of protein and a nucleic acid. (Note: Although these proteins contain phosphorus, its combination is different from that of the phosphoproteins. In the same way, the carbohydrate molecule present here is in different combination from that of the glycoproteins.) Examples: Nuclein; nucleohistone; (the chromatin material).

III. *Derived Proteins.*—Members of this group are products of enzyme action or the resultant of chemical or physical changes upon native proteins. This accounts for the modification of their properties.

A. *Primary Derived Proteins.*—Those products which are modified the least.

a. Proteans.—The first products formed when the protein is subject to the action of enzymes, acids, or in some cases, water. They are insoluble in water. Examples: Edestan, myosan.

b. Metaproteins.—Products obtained by a little longer continued action of acid or alkalies. Properties: weakly acid or basic; insoluble in water. Examples are acid or alkali albuminate.

c. Coagulated proteins.—Proteins rendered insoluble by the action of alcohol or heat. Examples: Egg albumin to which alcohol has been added—or heat coagulated.

B. *Secondary Derived Protein.*—Highly modified.

a. Proteoses.—Hydrolytic cleavage products of proteins. They are soluble in water. Non-coagulable by heat. Precipitable when their solutions are completely saturated with ammonium sulphate.

b. Peptones.—Hydrolytic cleavage products of proteins. Soluble in water. Non-coagulable by heat. Not precipitable by saturating their solutions with ammonium sulphate. Usually diffusible—give biuret reaction.

c. Peptides.—Compounds of amino acids of known composition. May or may not give a positive biuret test. Not coagulable by heat.

78.—Elementary Composition of Proteins

Detection of Nitrogen by Lassaigne's Method. Also Test for Sulphur and Phosphorus.

(a.) Place a little dry powdered protein (a mixture of casein and dry egg albumin) into a dry cheap test tube. Add a piece of metallic sodium the size of a pea. Then gradually heat to redness, maintaining at this temperature for a short time. Allow to cool. The heated end of the tube is broken into an evaporating dish containing 2 to 3 cc. of water. Cover immediately with a moist filter paper. After five minutes cautiously add 25 cc. of water. Stir well and filter the solution into a test tube. Divide the filtrate into three portions. This solution contains sodium cyanide, sodium sulphide and a sodium phosphorus compound, together with some sodic hydrate.

(b.) To test for nitrogen add to one portion a few drops of a fresh ferrous sulphate solution and then a drop or two of ferric chloride solution. Acidify slightly with hydrochloric acid. The hydrochloric acid dissolves the precipitated ferrous and ferric hydroxides. If sodium cyanide is present there remains a blue precipitate, due to the formation of Prussian Blue.

(c). Test another portion for phosphorus in the following manner: Acidify a small portion with nitric acid and add twice its volume of ammonium molybdate solution $(NH_4)_2MoO_4$.

Heat gently for a minute. Allow the solution to stand if no precipitate forms at once. The yellow precipitate is ammonium phospho-molybdate of a complicated and somewhat variable composition. (Approximately $(NH_4)_3PO_4 \cdot 10MoO_3$).

(d.) To another portion test for the presence of sulphur by adding 5 drops of concentrated sulphuric acid to the liquid in the test tube (or sufficient to make it strongly acid), and suspend a piece of filter paper previously moistened with lead acetate solution over the mouth of the tube, heat gently if necessary. The lead acetate turns dark. This is due to the formation of lead sulphide by the hydrogen sulphide, which is liberated from the liquid in the test tube.

Sulphur in Protein

Sulphur is believed to be present in two different forms in the protein molecule. The first form, which is present in greatest amount, is loosely combined with carbon and hydrogen. Sulphur in this form is variously termed "unoxidized, loosely combined, mercapton, and lead blackening sulphur." The second form is combined in a more stable manner with carbon and oxygen and is known as oxidized or acid sulphur. The protamines are the only class of sulphur-free proteins.

Test for Loosely Combined Sulphur

(e.) Put a little protein (wool) into a test tube with a few cc. of strong sodium hydrate solution. Add 2 or 3 drops of lead acetate solution and boil. Loosely combined sulphur is indicated by a darkening of the solution, the color deepening to black if sufficient sulphur is present. Acidify the solution with sulphuric acid and note the characteristic odor which is evolved from the solution. Explain the chemistry of this reaction.

Test for Total Sulphur in the Molecule

(f.) Boil some protein with 10 cc. of strong nitric acid in an evaporating dish in a hood, taking care not to char the organic matter. When a clear solution is obtained add 10 cc. of strong hydrochloric acid and evaporate to one-third of its volume. Add 25 cc. of water and test the solution for sulphates by means of barium chloride and for phosphorus as in the above experiment, (c).

(g.) Test protein for carbon and hydrogen, using the methods employed under carbohydrates.

Note: The protein color reactions and precipitation tests can best be illustrated in the laboratory by the use of a dilute solution of egg albumin. As egg white contains about 12% of albumin, it is best to dilute it with five volumes of distilled water. The egg white should be thoroughly agitated with the water, preferably by shaking in a corked bottle until uniform. It must be filtered through a plug of absorbent cotton until clear or at least nearly so. The filtrate is then suitable for use.

79.—Color Reactions

A. Millons Reaction:

Place 5 cc. of egg albumin solution in a test tube and add a few drops of Millon's reagent. A white precipitate forms, which upon heating should turn red.

Note: The color reaction is due to the presence of the hydroxyl-phenyl group (C_6H_4OH) in the protein molecule. Certain non-proteins (compounds) such as tyrosine, carbolic acid and thymol respond to this reaction. Inasmuch as tyrosine contains the only hydroxyl-phenyl group which has been positively proved to be present in the protein molecule, it is evident that the proteins which respond to Millon's reaction must contain this tyrosine complex. On account of the nature of the reagent this test is extremely unsatisfactory in the presence of certain inorganic salts, e.g. sodium chloride.

B. Xanthoproteic Reaction:

To 2 to 3 cc. of egg albumin solution in a test tube add concentrated nitric acid. A white precipitate forms, which upon heating turns yellow and finally dissolves, imparting a yellow color to the solution. Cool the solution and carefully add an excess of ammonium or sodium hydroxide. The color deepens into an orange.

(Note: This reaction is due to the presence in the protein molecule of the phenyl group which, with nitric acid, forms certain nitro derivatives. The complexes in the protein molecule responsible for this reaction are tyrosine, phenylaline and tryptophane.)

C. Hopkins-Cole Reaction:

To 1 or 2 cc. of egg albumin solution in a test tube add 3 cc. of glyoxylic acid solution ($CHO.COOH-H_2O$). Carefully pour 5 cc. of concentrated sulphuric acid down the side of the inclined test tube At the junction of the two liquids a reddish-violet color is formed. This is due to the presence of the tryptophane group. This test is not given in the presence of oxidizing agents.

(Note: Glyoxylic acid or "reduced oxalic acid" is prepared by treating half a liter of a saturated solution of oxalic acid with 40 grams of 2% sodium amalgam in a tall cylinder. When all the hydrogen has been evolved, the solution contains oxalic acid, sodium binoxalate and glyoxylic acid. It is kept in a closed bottle containing chloroform. Benedict recommends reducing the oxalic acid by means of powdered magnesium. 10 grams of powdered magnesium are suspended in 100-150 cc. of water in a 1½ liter pyrex flask. Have a large dish of cold water available in which the flask may be placed for cooling. Now add 250 cc. cold saturated oxalic acid; slowly, and while rotating the flask. Keep the temperature down by setting the flask into the cold water. When the reaction is complete, filter off the precipitate of magnesium oxalate; acidify the filtrate with acetic acid and dilute with distilled water to 1 liter. Preserve as indicated above.)

D. Biuret Test:

To 2 to 3 cc. of egg albumin solution in a test tube, add an equal volume of concentrated (40%) potassium or sodium hydroxide solution, mix thoroughly, and add, drop by drop, a very dilute (1%) copper sulphate solution, until a purplish-violet or pinkish-violet color is produced.

(Note: The depth of the color depends upon the nature of the protein. Proteoses and peptones give a decided pink color, while gelatin gives a color which is not far removed from blue. All substances containing any of the following amino groups: $-CONH_2$: $-CSNH_2$ $-C(NH)NH_2$; or $-CH_2NH_2$; repeated at least twice in the molecule directly linked together, or

when linked by means of a third carbon atom or nitrogen atom will give the biuret test. This test is given by such compounds as oxamide, malonamide and biuret, representing some of the types of linkages referred to above.)

The biuret reaction alone is therefore no proof as to the protein nature of a substance. Even substances like urobilin give a color hardly distinguishable from that given by proteins with this test. Proteins can be modified by treatment with nitrous acid, and rendered incapable of giving the reaction.

No protein reaction is in itself characteristic, and therefore, in testing for proteins, one reaction is not sufficient. A number of precipitation tests and color reactions must be employed.

Influence of Ammonium Sulphate Upon the Biuret Test

Repeat the biuret test, after adding to the albumin solution some ammonium sulphate. How is the color modified? Repeat the test with the ammonium sulphate, adding a large excess of 40% alkali. What is the practical bearing of this experiment?

E. Liebermann's Reaction:

Place 5 cc. of concentrated hydrochloric acid in a test tube, and add a little dry egg albumin or a small piece of the white of a hard boiled egg. Boil the mixture until a pinkish-violet color results. If the protein is boiled with alcohol, then washed with ether previous to making the test, a deeper blue color is obtained. This test is indicative of tryptophane in the protein molecule.

F. Molisch's Reaction:

Place 2.5 cc. of egg albumin in a test tube. Dilute with an equal volume of water. Add 3 to 4 drops of Molish reagent, (1% solution of alpha napthol in alcohol) and mix. Incline the test tube and allow 5 cc. of concentrated sulphuric acid to run down the side of the tube and stratify under the albumin solution. A violet ring, forming at the junction of the fluids should be observed. This reaction is dependent upon the presence of a carbohydrate group in the molecule. In certain proteins this group is glucosamine. Compare with Experiment 30, page 42, carbohydrates.

G. Sulphur Reaction:

The test for lead blackening sulphur, page 104,-e, under "Elementary Composition," above, indicates the presence of cystein or cystin in the protein molecule.

80.—Precipitation Tests

(a.) "Salting Out" by Neutral Salts.

1. Precipitation by Ammonium Sulphate.

Add some solid ammonium sulphate to 10 cc. of the albumin solution in a test tube, shaking frequently, until the solution is thoroughly saturated. Allow to stand for a while, shaking occasionally. Then filter, and test the filtrate for albumin by means of the heat test. Test the solubility of the precipitate remaining on the filter paper.

2. Precipitating Action of Magnesium Sulphate.

Perform a similar experiment, using solid magnesium sulphate instead of ammonium sulphate. To a portion of the filtrate add 1 or 2 drops of acetic acid. Heat.

(Note: Magnesium sulphate does not precipitate albumin upon saturation of its solution. A closely related protein called globulin may be distinguished from albumin by reason of its behavior with this salt.)

(b.) Precipitation by Mineral Acids.

Nitric Acid Test

Put 5 cc. of the albumin solution into a test tube and introduce 5 cc. of concentrated nitric acid (very carefully by means of a pipette) into the bottom of the test tube. This forms a layer under the albumin solution at the junction of which a white ring is seen. Determine the lowest protein concentration at which this test is unmistakable. Allow ten minutes if the reaction is slow in appearing. This test is clinically known as "Heller's Ring Test," when applied to urine analysis. Sulphuric acid, hydrochloric acid, and meta-phosphoric acid are also capable of precipitating protein. Orthophosphoric acid will not cause precipitation. Why?

(c.) Precipitation by Alcohol.

Add an excess of alcohol (1 or 2 volumes) to 5 cc. of the albumin solution.

(d.) Coagulation Test.

Heat 5 cc. of albumin solution in a test tube to boiling. Note the degree of coagulation. Now add from 1 to 3 drops of 0.5% acetic acid and boil again. Complete coagulation should occur.

(e.) Precipitation by Metallic Salts.

1. To 5 cc. of albumin in a test tube add 5% mercuric nitrate solution drop by drop, until a definite precipitate is obtained. To the solution containing the precipitate, add saturated sodium chloride, a few drops at a time. What happens? Is there any difference in degree of dissociation of mercuric nitrate and mercuric chloride? Explain.
2. Repeat the above experiment with a 5% ferric chloride solution. Omit the addition of sodium chloride.
3. Repeat with solutions of any lead, silver and copper salts which are in the laboratory. Record your results.

(f.) Precipitation by "Alkaloidal Reagents."

Most of these reagents acts upon protein in acid solutions (peptones are exceptions in some cases). The protein under these conditions possesses a positive charge. The large negatively charged cation of the precipitating reagent is believed to be adsorbed by the protein, precipitation resulting.

1. Tannic Acid.

   Make 5 cc. of the albumin solution acid with dilute acetic acid, then add a few drops of a tannic acid solution.

2. Picric Acid Test (Esbach's Reagent).

   Add to a portion of the albumin solution a few drops of Esbach's reagent. Esbach's reagent is a dilute solution of picric acid containing some citric acid. De-

termine the lowest protein concentration at which this test is unmistakable.

3. Acetic Acid and Potassium Ferrocyanide.

Make a portion of the albumin solution strongly acid with acetic acid, and add a few drops of potassium ferrocyanide solution. A white flocculent precipitate is formed (not with peptones).

4. Phospho-tungstic Acid.

Acidify 5 cc. of the albumin solution with dilute hydrochloric acid, and add a few drops of the phospho-tungstic acid reagent. This is a very delicate test.

5. Potassio-mercuric-iodide (Brück's Reagent).

Acidulate 5 cc. of the albumin solution with dilute hydrochloric acid, and add a few drops of potassio-mercuric-iodide solution.

Note.—Brück's reagent is prepared by dissolving 50 grams of potassium iodide in 500 cc. of distilled water. To this solution add 120 grams of mercuric iodide, filtering if necessary to remove any undissolved material. The solution is usually diluted to 1 liter, but may be used without dilution if advisable.

6. Sulphosalicylic Acid.

To 5 cc. of albumin solution add a few drops of a 20 per cent. solution of sulphosalicylic acid.

Note: To prepare sulphosalicylic acid, dissolve 13 grams of salicylic acid in 20 grams of warm sulphuric acid. When dissolved, cool, and pour into 67 cc. of distilled water.

7. Metaphosphoric Acid.

To 5 cc. of albumin solution add drop by drop a 25 per cent. solution of metaphosphoric acid. This solution must be freshly prepared, and without the aid of heat.

81.—Properties of Individual Proteins

Simple Proteins

(Substances yielding only alpha-amino acids or their derivatives on hydrolysis)

(a.) Albumins. Coagulation by Heat.

1. Heat a little of the solution in a test tube. Compare the coagulation so obtained with that obtained when the solution is diluted:
   (a) 10 times with distilled water.
   (b) 20 times with distilled water.
   (c) 20 times with 0.1% salt solution.
   (d) 20 times with 0.01% acetic acid.
   (e) 20 times with equal volumes of 0.1% salt solution, and 0.01% acetic acid solution. From the above tests, what do you conclude are the necessary factors for coagulation?

2. Ascertain the temperature of coagulation of albumin as follows: Faintly acidify a portion of the solution with a few drops of 0.5% acetic acid. Filter if necessary. Place the solution in a test tube, insert a thermometer by means of a cork, and suspend the tube in a large beaker of water. Heat the beaker slowly with a small flame, and observe the point at which the albumin becomes cloudy.

3. Include all tests in Experiment 80 above as part of this division also.

(b.) Globulins.

Globulins are simple proteins and are especially predominant in the vegetable kingdom. They are closely related to the albumens, and consequently react to all the general precipitants and color tests. Globulins are insoluble in pure water, thus differing from albumins. They are soluble in neutral salt solutions, which are not too concentrated. Most globulins are precipitated from their solutions by saturation with solid sodium chloride or solid magnesium sulphate. Ammonium sulphate at *half saturation* precipitates globulins. As a class, they are less

stable than the albumins. Upon repeated precipitation, globulins dissolve with greater difficulty. The following tests serve to distinguish globulins from other proteins. These tests are made upon blood serum, which contains an albumin as well as globulin. At least two and possibly more globulins are present in blood serum. Euglobulin is fractionated out most easily, being precipitated by ammonium sulphate when the concentration of the salt has reached 28 to 36 per cent. The second fraction called pseudoglobulin comes out between 36 and 44 per cent of saturation. Each fraction probably represents a number of globulins rather than a single definite compound.

1. Coagulation by Heat.

   Globulins, like albumins, are coagulated by heat. They are distinguished from other proteins and from one another by the temperature at which they coagulate. To test this upon serum globulin, proceed as follows: To 10 cc. of serum, add an equal volume of a saturated solution of ammonium sulphate, thus obtaining a half saturated solution. The bulky precipitate is globulin. Filter off the precipitate, wash two or three times with a half saturated ammonium sulphate solution, and dissolve in 20 cc. of distilled water. This will give you a clear solution of globulin. As stated above, the pure globulin does not dissolve in water, but in this case, the presence of salts, used for precipitation and held mechanically in the precipitate, is sufficient to cause it to dissolve. Test the temperature of coagulation of this solution with the same apparatus that you used for egg albumin. What is the difference?

2. Action of Carbon Dioxide.

   Dilute 5 cc. of serum with 45 cc. of ice cold water. Place the mixture in a cylinder, surrounded by ice water, and pass through it a stream of carbon dioxide. A flocculent precipitate is obtained. Continue the carbon dioxide for some time and notice its effect upon the precipitate. Explain to the instructor.

3. Precipitation by Dialysis.

Pour 20 cc. of serum into a parchment dializing tube, previously soaked in distilled water. Suspend the tube with its contents in a large vessel of distilled water, changing the water at frequent intervals, (one hour at first). After 24 hours, examine the serum in the dializing tube. The flocculent precipitate is globulin. Explain to the instructor the cause of this precipitation.

4. Precipitation of Euglobulin by Dilution.

Slightly acidify 5 cc. of serum with dilute (0.5%) acetic acid. Pour the serum, drop by drop, into a graduate filled with distilled water. What takes place? Explain. Allow to stand a few minutes.

5. Precipitation by Magnesium Sulphate.

Saturate 5 cc. of serum with solid magnesium sulphate. A heavy precipitate will be formed. Compare this with the action of the same salt on a solution of egg albumin. The globulin is so completely precipitated by this salt that the method may be used for its quantitative estimation.

6. Edestin.

The preparation of this vegetable globulin is given on page 94.

(c.) Glutelins and Prolamines.

Osborne has shown that seeds of cereals, after extracting with water and with a dilute solution of a neutral salt, still contain protein. One of these proteins may be removed by 80 per cent alcohol. He designates this group as prolamines, because upon hydrolysis of these compounds, especially large amounts of prolein and ammonia are obtained.

The following prolamines have been described by Osborne:

Zein from maize
Hordein from barley
Gliadin from wheat and rye
Bynine from malt.

If the residue remaining after the alcohol extraction of prolamines be treated with dilute acid or dilute alkali, another protein is recovered. Members of this group are called glutelins. Glutenin, from the wheat is an example of this class.

1. Extraction of Gliadin.

   Mix some flour into a thick paste with warm water, and tie up in a piece of cloth. Work this paste under tepid water, changing it frequently until the washings are free from starch. What remains? Carefully grind up the residue in a mortar, containing a little sand, with 5 portions of 70% alcohol, using about 25 cc. each time. Save the alcohol washings and the residue insoluble in alcohol. The alcoholic solution will contain gliadin. Filter.

2. Protein Color Reactions with Gliadin.

   Dilute the filtrate with water. What happens? Try all protein color reactions. *Caution*: Alcohol is oxidized by concentrated nitric acid. Add small amounts of acid at a time—cool continuously under the water tap.

3. Glutenin.

   Test the solubility of the residue which consists mainly of glutenin. With a solution of glutenin, perform the protein color reactions.

(d.) Albuminoids.

The members of this group are important constituents of the animal skeleton or cutaneous structure. As a rule, they all occur in an insoluble state in the organism, and uniformly resist solution by ordinary protein solvents as well as by all except the most vigorous chemical reagents. They are therefore characterized by their inactivity.

Among the members of this class may be mentioned the keratins, elastin, collagen, reticulin and others.

1. Keratin.

   The albuminoid, keratin, makes up the major part of hair, wool, horn, hoof, feathers, nails and the epi-

dermal layer of the skin. Keratins, as a group, possess very similar properties. They are insoluble in the usual protein solvents, and are not acted upon by the gastric or pancreatic juices. They respond to Millon's reaction and Xanthoproteic reaction, and are characterized by containing large amounts of loosely held sulphur. Pure keratin may be prepared by successive treatment with artificial gastric juice, artificial pancreatic juice, boiling alcohol and boiling ether. Each process should last for at least 24 hours.

Using scoured wool, test the solubility of keratin in the ordinary protein solvents. Perform the protein color reactions upon small amounts of wool. (Do not try to put the wool into solution before performing the tests.)

2. Collagen and Geletin.

Collagen, or gelatin-forming substance, occurs extensively in vertebrates. It is the chief constituent of the fibrils of the connective tissue (as ossein) and of the organic substances of the bony tissue. By continued boiling with water, especially if a little acid is present, collagen is converted into gelatin. Gelatin is of somewhat variable composition, depending upon the collagen, from which it was made.

Gelatin solutions are not precipitated on boiling or by mineral acids, acetic acid, alum, basic lead acetate, or metallic salts. They are precipitated when acidified with acetic acid by potassium ferro-cyanide on careful addition of the reagent. Gelatin is precipitated by tannic acid in the presence of salts, by acetic acid and sodium chloride; by mercuric chloride in the presence of hydrochloric acid and sodium chloride; by metaphosphoric acid; phosphomolybdic acid in the presence of hydrochloric acid; also by alcohol, especially in the presence of neutral salts. The biuret reaction is positive, but the Hopkins-Cole reaction is negative. Millon's reaction and the xanthroproteic reaction, when given, are probably due to impurities.

(a.) Preparation of Gelatin.

Carefully clean the shaft of a long bone. Break or cut to small bits, then weigh out a sample of about 10 grams. Place the material in a flask and cover with about 60 cc. of dilute hydrochloric acid (1-20). Allow the material to remain for three or four days. Dilute hydrochloric acid dissolves the inorganic portions of the bone, leaving the collagen. Note the effervescence due to the presence of carbonates. The acid solution is poured off. Save for Exp. 101, page 134. The remains of the bone are allowed to stand over night in a dilute solution (1-10) of sodium carbonate, then boiled in 100 cc. of water for one or two hours. The collagen undergoes hydration, and is converted into the soluble substance gelatin. The core of bone untouched by the acid usually remains. Evaporate the solution to 25 cc. volume, and allow to cool. A firm jelly should form if the solution is sufficiently concentrated. If the solution gelatinizes, add an equal volume of water and heat again. This solution may be used for the following tests. If the solution is not pure, it is advisable to prepare one from commercial gelatin.

(b.) Precipitation Tests and Color Reactions.

With a gelatin solution perform protein precipitation and color tests. Tabulate your results, and explain them to the instructor.

Conjugated Proteins

(Compounds of simple proteins and some other non-protein group. The non-protein group is usually acid in nature.)

(a.) Chromoproteins.

Haemoglobin is an example of this group and is composed of a nonprotein pigment substance which contains iron (haematin) and the protein globin, which belongs to the class of histones.

Hemoglobin is soluble in water and dilute salt solutions. It is decomposed by acids and alkalies into its constituents. Heat

causes it to coagulate. It is generally insoluble in organic solvents.

Work upon the compound will be given later under blood.

(b.) Glycoproteins.

Glycoproteins are compound proteins, which yield upon decomposition a protein substance, on the one hand, and carbohydrates or derivatives of these on the other, but no purine bodies. Certain glycoproteins contain no phosphorus. These are mucin substances, chondroproteins and hyalogens. Others contain phosphorus (phosphoglyco-proteins). The glycoproteins yielding no phosphorus may be divided into two groups respective to the carbohydrate radical split off. These are mucin substances and chondroproteins. Mucins yield upon cleavage an amino-sugar, which in most cases is glucosamine. Chondroproteins, on the other hand, yield upon cleavage a protein and chondroitin-sulphuric acid.

1. Preparation of a Mucin.

   Extract an umbilical cord for twenty-four hours with 10% barium hydrate solution in a stoppered flask. Filter. To the filtrate add acetic acid, drop by drop, which precipitates the mucin. Allow to settle, filter, and wash with water. Mucins may also be prepared from saliva by precipitation with acetic acid.

   a. Try the solubility of mucin in water, potassium hydrate, calcium hydrate solution, and in a 5% hydrochloric acid.

   b. Perform the protein color tests with mucin.

   c. Boil mucin for some time with dilute hydrochloric acid (1-3), allow to cool, neutralize with sodic hydrate solution and test for a reducing sugar with Fehling's solution. Where did the reducing carbohydrate come from?

(c.) Phosphoproteins.

This group of substances contains the protein molecule and some, as yet undefined, phosphorus-containing substances *other*

than a nucleic acid or lecithin. They occur extensively in the animal and plant kingdoms. Casein of milk is the best studied phosphoprotein. Lecithalbumins occur in ovovitellin. In this body they are supposed to occur as a combination of protein and lecithin. Similar substances also occur in fish eggs. These substances containing lecithin can be removed only with difficulty or incompletely from proteins by a mixture of alcohol and ether.

1. Using Casein as an example of this class, perform the following experiments:
   a. Solubility in water, acids and alkalies.
   b. Protein color reactions.
   c. Test for phosphorus. (Note: To do this, fuse a little in a crucible with sodium carbonate and sodium nitrate. Dissolve the fused mass, when cold, in water, and test for phosphoric acid by means of ammonium molybdate.)
   d. Digest a portion for some time at 40°C. with pepsin-hydrochloric acid. Filter and test the filtrate for proteins. What is the insoluble residue?

(d.) Nucleoproteins—Nuclein—Nucleic Acid.

Note: The extended discussion of nucleoproteins contained in this section is admittedly out of place in a laboratory manual of this type. It is inserted with the hope that the facts presented will clarify the student's mind after reading the section upon this subject in the text-book.

The nucleoproteins are compounds of nucleic acid with a protein. Typical nucleoproteins are characteristic constituents of the cell nuclei.

Nucleoproteins are insoluble in water, soluble in dilute alkali, from which they may be precipitated by carbon-dioxide or dilute acetic acid.

From the researches of Hammarsten (1894) two nucleoproteins are recognized as extractable from glandular tissue.

Hammarsten gave to these the names of α and β—nucleoprotein. The α-nucleoproteins can be prepared from the cloudy aqueous gland extracts, by precipitation with acetic acid. The α-nucleoprotein consists of various salts of protein with nucleic acid in which the protein is in excess. Upon digestion with pepsin-hydrochloric acid part of the protein is digested away, leaving a mixture of protein and acid salts. This indefinite mixture is called "nuclein." It is resistant to the further action of pepsin-hydrochloric acid.

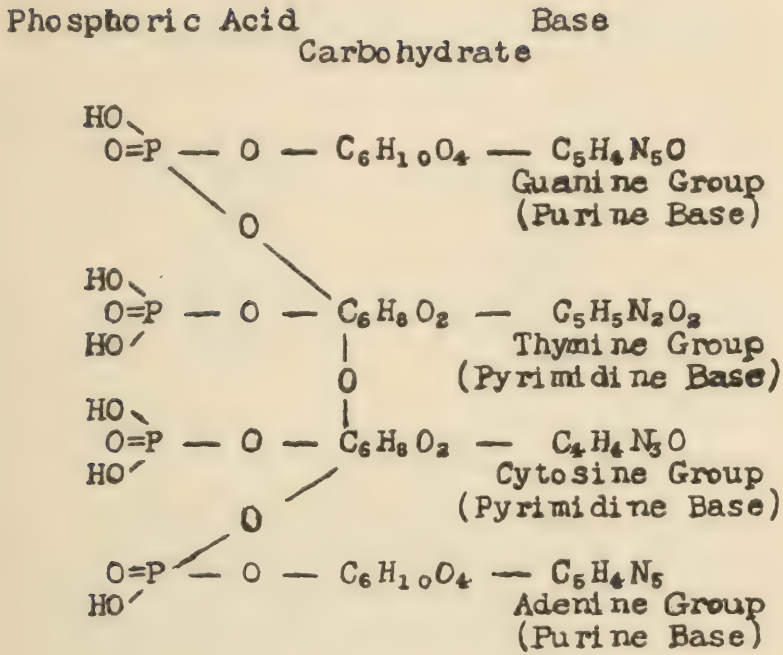
The β-nucleoprotein is obtained by rapidly heating a suspension of ground tissue in water to boiling, filtering off the coagulum, and then upon the addition of acetic acid to the filtrate, a flocculent precipitate is obtained. The α-nucleoprotein remains in the heat coagulum in this case. Jones states that there "is no evidence to show that β-nucleoprotein is a constituent of the cell nucleus."

Nucleoproteins are of special interest on account of the nucleic acids which they contain. Nucleic acids are split down by acid hydrolysis into three essential constituents. These are:

1st, phosphoric acid; 2nd, a carbohydrate radical (hexose or pentose), and 3rd, a nitrogenous base or bases belonging to either the group of purine bases or pyrimidine bases.

The nucleic acids derived from α-nucleoproteins, will yield upon complete hydrolysis two purine bases, and also two pyrimidine bases in addition to the phosphoric acid and the carbohydrate.

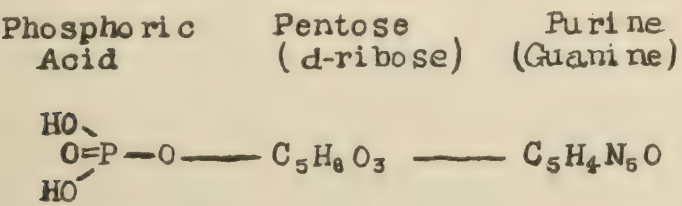
The formula given on following page, shows the present state of our knowledge as to the composition of a nucleic acid.



Thymus Nucleic Acid.

From the structure of the thymus nucleic acid, it is seen that two types of linkages between nucleotids occurs. Jones believes all four parts of *yeast nucleic acid* to be linked by ties similar to that connecting the thymine group to the cytosine group in the above formula. Nucleic acids containing four parts as indicated, occur in ∝-nucleoproteins. Therefore, ∝-nucleoproteins are compounds of protein with a tetranucleotid.

The *β*-nucleoproteins, on the contrary, are compounds of protein with an acid "guanylic acid," which is a mono-nucleotid. One of the possible formulae for this nucleic acid is given below:



Guanine mono-nucleotid.

The following table from Jones, gives a general idea of the composition of nucleic acids from nucleoproteins.

Hydrolytic Products of Nucleic Acids

	Of Plant Origin	Of Animal Origin
	1. Phosphoric Acid	1. Phosphoric Acid
Purine Bases	2. Guanine	2. Guanine
	3. Adenine	3. Adenine
Pyrimidine Bases	4. Cytosine	4. Cytosine
	5. Uracil	5. Thymine
	6. Pentose	6. (Hexose) Laevulinic Acid

Jones furthermore suggests that the animal nucleic acids of various origin are identical with one another.

1. Preparation of Thymus Nucleic Acid (Jones)

To a boiling mixture of 2 liters of water, 100 grams of sodium acetate and 33 grams of caustic soda in a flask, add in small successive portions, 1000 grams of trimmed and finely ground thymus gland. The tissue should dissolve quickly, if not, heat for a short time over a free flame. Immerse the flask in a briskly boiling water bath for 2 hours, stirring occasionally. Now dilute with 1/3 volume of hot water, and faintly but distinctly acidify with 50% acetic acid. Usually 100 cc. acid is required, the final addition should, however, be carefully controlled by testing with litmus paper. If too much acid is added, it should be neutralized by addition of dilute sodium hydroxide. Small portions should from time to time during neutralization be tested for filterability. When the acidity favorable for rapid filtration has been obtained, heat the material to hard boiling, then filter through paper using a hot water funnel. The filtration should be fairly rapid, a green slime remaining on the paper. The filtrate will gelatinize upon cooling. Evaporate the filtrate to about 750 cc. on the steam bath. Pour the hot concentrated solution slowly into 1000 cc. of 95% alcohol. Allow to stand over night. Sodium nucleate settles out as a

white spongy mass, from which the brown fluid should be decanted and the remaining portion pressed out with a spatula, leaving the material in one cohesive mass.

Wash the residue of sodium nucleate by decantation respectively with 80% and 95% alcohol. Press out as thoroughly as possible after the last treatment with alcohol. Now add 300 cc. of boiling water, transfer to a suitable flask, and heat on the water bath. In half an hour or less, insoluble phosphates will collect, leaving a perfectly transparent interstitial fluid. To lower the viscosity so that the fluid will filter readily, 10 cc. of 20% caustic soda are added, and after thoroughly mixing, the solution is filtered, using a hot water funnel. The clear filtrate is acidified with acetic acid, then poured into 700 cc. of 95% alcohol. Sodium nucleate is precipitated as before, then washed with 80, 90 and 95% alcohols, finally transferring to a mortar, where it is ground to a fine white powder under absolute alcohol. The ground mass is now washed onto a filter with absolute alcohol, drained thoroughly and transferred to a watch crystal and placed in a desiccator over sulphuric acid until thoroughly dry. Yield about 33 grams.

This is the sodium salt of thymus nucleic acid. it should be fine and white, and not hygroscopic. This method is applicable to the preparation of similar products from the pancreas and spleen.

Properties: A 4 per cent. solution of sodium nucleate in warm water becomes gelatinous when cooled to the room temperature. With this solution or one slightly more concentrated, the following properties may be demonstrated.

a. The viscosity of the fluid is decreased by the addition of either acetic acid or sodium hydroxide. The solution may be changed back and forth from gelatinous to fluid by the alternate addition of acid and alkali.

b. The positive optical rotation is greatest in a neutral fluid, and like the viscosity, is markedly lowered by the addition of either acid or alkali.

c. The specific rotation falls by dilution with water, but more rapidly by dilution with acid or alkali.

d. The rotation is lowered by heating a solution from 20° to 40°C., but again rises when the solution cools.

Derived Proteins

Products resulting from chemical or physical changes, or the action of enzymes upon native proteins.

(a.) Primary Derived Proteins.

1. Proteans.

If edestin (globulin, see page 84) be prepared by extracting crushed hemp seed with 5% warm (60°C.) sodium chloride for one-half hour, then filtered, and filtrate allowed to cool very slowly (this may be obtained by placing the filtrate contained in a flask into an earthen ware jar or crock, filled to the proper height with water at 60° C., then allowing this system to cool) beautiful crystals of edestin separate out. Filter off the edestin.

Dissolve some of the edestin (globulin) in a 0.2% hydrochloric acid, allowing it to stand for one-half hour. The solution must now be neutralized with 0.5% sodium carbonate, and the precipitate which is *edestan* (protean) filtered off.

With the precipitate try the solubility in water, sodium chloride, and dilute acid and alkali.

Apply the protein color reactions and do the protein precipitation tests.

How does this protein differ from a globulin or albumin?

2. Metaproteins.

There are two principal metaproteins, the acid metaprotein (so called acid albuminate) and the alkali

metaprotein (so called alkali albuminate). Aside from solubilities, these two types differ in their nitrogen and sulphur content when made from the same protein. The acid metaprotein contains more of these elements, as loss occurs due to the action of the alkali used in forming alkali metaproteins.

To prepare an alkali metaprotein from albumin, proceed as follows:

Separate the white from the yolk of an egg, and place it in an evaporating dish. To it add 40% potassium hydroxide solution, DROP BY DROP, stirring continuously until the albumin has attained the consistency of a jelly. This is known as Lieberkühn's jelly, and is the desired alkali metaprotein.

The jelly may now be cut into thin strips, and the alkali leached out by covering and decanting the material with distilled water several times.

Place some of the material in a beaker, add sufficient water to cover it and then heat gently. If the material fails to dissolve note the fact, and then add a drop or two of alkali. When in solution, cool and divide into two parts. With the first part do the protein precipitation tests and the biuret reaction. To the second part, add dilute hydrochloric acid until neutral, noting the odor of hydrogen sulphide emitted. Filter off the precipitate, wash with water, and using the solid material perform the protein color reactions, including the test for lead blackening sulphur.

3. Coagulated Proteins.

With pieces of the white of a hard boiled egg, perform solubility and color tests.

(b.) Secondary Derived Proteins.

1. Proteoses and Peptones.

There are several proteoses (protoproteose, heteroproteose and deuteroproteose) resulting from proteolysis. They are differentiated mainly by their solubili-

ties in salt solutions, especially in ammonium sulphate solutions of varying concentrations.

The "peptones" sold for bacteriological purposes contain a large amount of proteose.

It is impossible to take up the proteoses without defining their properties in relation to the peptones. There are at least two peptones recognized, the amphopeptone, and the antipeptone.

The proteoses as a class represent protein cleavage products less completely digested than the peptones. Neither proteoses or peptones are coagulable by heat. They both are extremely soluble in water, and diffuse rapidly, the peptone having even greater diffusion than the proteoses. Peptones differ from proteoses chiefly by the fact that they are non-precipitable by ammonium sulphate, give no precipitate with potassium ferrocyanide and acetic acid, potassium mercuric iodide and hydrochloric acid, picric acid, and trichloracetic acid.

Peptones are precipitated by phosphotungstic acid, phosphomolybdic acid, absolute alcohol, and tannic acid. Many of the precipitants when added in excess dissolve the precipitate.

(a.) General Properties of Proteoses and Peptones.

Using Witte's or some other commercial "peptone" try the solubility in various solvents (water, alcohol, dilute acid and dilute alkali) and with a solution perform the protein color reactions.

(b.) Separation of Proteoses from Peptones by Means of Ammonium Sulphate.

Weigh out 5 grams of Witte's or another commercial peptone, and make a solution with a small amount of hot water (50 cc. if possible). To this add an equal volume of saturated ammonium sulphate (thereby giving you a half saturated solution). A precipitate of primary proteoses (protoproteose and heteroproteose) should be observed. Place the solution in a pyrex beaker, and heat to boiling. Add solid ammonium suplhate until

the solution becomes saturated. The secondary proteose (deuteroproteose) now separates out and the solution contains the peptone. If a rubber tipped stirring rod be worked about in the beaker, the sticky proteose may be collected and removed. Dissolve the proteose in water in a beaker, precipitate the ammonium sulphate by adding barium carbonate, boil, filter, then concentrate the filtrate upon the steam bath. With the concentrated filtrate, perform the protein precipitation tests.

The saturated ammonium sulphate solution containing the peptone should be cooled, and filtered through cloth. A portion of the filtrate is diluted with water, barium carbonate added as above, boiled, filtered, and the filtrate concentrated upon the steam bath. With the concentrated solution so obtained repeat the protein precipitation reactions, and compare with those given by the proteose solution above.

82.—Problem. Obtain two unknown proteins from the instructor. Run through the color reactions and precipitation tests and report to the instructor the class and name of the protein found. In some cases the student may receive a sample containing more than one protein. Identify and report each.

AMINO ACIDS

These are some of the ultimate cleavage products of the protein molecule.

83.—A. Synthesis of Glycocoll

Dissolve 20 grams of monochloracetic acid (CH_2Cl-$COOH$) in a flask by means of 50 cc. of cold concentrated ammonia. Cool with ice and saturate the solution with ammonia gas, shaking constantly in ice water to avoid any rise of temperature. Cover the flask with a watch crystal and allow to stand over night in a basin of cold water. This prevents any appreciable rise in temperature, due to the completion of the chemical reaction.

Take the flask out of doors and pour the contents into a large evaporating dish and leave for twenty-four hours to allow the escape of ammonia. At the end of this time, gently boil the contents of the dish until all the free ammonia is gone. Large amounts of cupric oxide will be dissolved, *if all of the*

ammonia is not expelled. Add freshly precipitated cupric oxide until some remains undissolved after five minutes boiling. (Cupric oxide is prepared by adding a slight excess of sodium hydrate to a dilute copper sulphate solution, and washing several times by decantation until the precipitate is free from alkali.) Filter the hot solution to remove the excess of cupric oxide. Evaporate the filtrate to a small volume (75-100 cc.) and allow it to cool. *Do not carry to dryness.* Upon standing overnight, an abundant precipitate of the copper salt of glycocoll should be obtained. Filter off these crystals on a Buchner funnel, and wash the precipitate successively with 60, 80, and 90 per cent. alcohol until the wash alcohol no longer shows a reaction for chlorine. (Note: Acidify the test portion with a few drops of nitric acid before adding the silver nitrate solution.)

To prepare free glycocoll from this salt, proceed as follows: Dissolve the copper salt in 150 cc. of hot water, then add a little freshly precipitated aluminum hydrate, and pass hydrogen sulphide gas into the liquid until saturated.

(Note: Aluminum hydrate is prepared by precipitation from aluminum sulphate by ammonia and washing by decantation until neutral. The hydroxide takes no part in the above reaction, but acts only to assist in the precipitation of the colloidal copper sulphide.)

Heat the liquid to boiling and filter. Concentrate the filtrate to about 20 cc. and precipitate the glycocoll by the gradual addition of alcohol. Filter the glycocoll on to a Buchner funnel, and wash once or twice with alcohol. Transfer the crystals to a watch glass, and place in a protected place to dry. Weigh the dry precipitate and calculate the yield in per cent of the theoretical. Determine the purity of the product by a nitrogen determination.

B.—Amino Acids by Acid Hydrolysis.

The preparation of cystine from wool: (Folin)

Put 50 grams of scoured wool into a 500 cc. Florence flask. Add 100 cc. of concentrated hydrochloric acid, and connect the flask with a condenser. (If a good hood is available, a two and one-half foot glass tube inserted through the cork serves as a

suitable air condenser.) Heat the flask in a water bath until the wool has been dissolved. Remove from the water bath, dry the flask thoroughly, boil gently over a very small flame until the biuret reaction is negative. Boil for one hour after the biuret reaction is entirely gone. This digestion requires from three to five hours. After completely digesting the mixture allow to cool, and add solid sodium acetate until no free mineral acid can be detected in solution by means of congo red paper. Explain the disappearance of mineral acid. Allow the mixture to stand 1 week. Filter the contents of the flask on a Buchner funnel, and wash once or twice in cold water. Dissolve the precipitate in 150 cc. of water, to which has been added 5 cc. (more if necessary) of concentrated hydrochloric acid and about 20 grams of purified bone black. Boil the mixture for 5 to 10 minutes. Filter again with a suction pump and collect the filtrate. The filtrate is heated to boiling and JUST neutralized by the addition of hot concentrated sodic acetate solution. Use congo red paper as an indicator. (AN EXCESS OF SODIC ACETATE MUST BE AVOIDED.) The precipitate formed consists of cystin and should be very white and pure. Examine a few crystals under the microscope and sketch their appearance. Filter them off after five minutes standing. Allow the liquid to cool. Tyrosin and leucin will crystallize out in the filtrate. Identify microscopically. Test the cystin crystals for sulphur by means of lead acetate and sodic hydrate.

(Note: To prepare pure bone black, the commercial sample is allowed to stand over night in an excess of dilute HCl. Then filter, and digest over a flame with a second portion of dilute hydrochloric acid. This process is repeated until the acid digestion mixture fails to give a test for phosphate. The charcoal is then thrown on to a large filter paper in a funnel, and washed free from chlorides.)

DIGESTION

The following experiments are intended to give the student an idea of the action of digestive ferments upon the foods taken into the body.

Salivary Digestion

84.—Collect about 50 cc. of saliva by chewing a piece of paraffine. Filter, and test the clear filtrate as follows:—

(a.) Reaction.—Test with litmus.

(b.) Mucin.—To 5 cc. add a few drops of dilute acetic acid and record the result.

(c.) Proteins.—Filter (b) to remove the mucin. Test the filtrate by the xantho-proteic test.

(d.) A sulphocyanide.—Add to some saliva a drop or two of very dilute and acidulated (HCl) solution of Fe_2Cl_6. Report the result. Then add a few drops of a solution of $HgCl_2$. What is the result? What other substances give a similar reaction with ferric chloride, and how may they be distinguished from each other and from sulphocyanic acid?

(e.) Action of saliva upon starch.—Take some filtered saliva in a test tube, and place in a water-bath at 40° C. for five or ten minutes. Put some starch paste into a second test tube, and place this also in the water-bath for a while, then mix the two (10 cc. of starch paste to 3 cc. of undiluted saliva) and return to the water-bath. The starch is changed first to soluble starch (if originally a thick paste, it becomes fluid and loses its opalescence), then to erythro-dextrin, which gives a red color with iodine, and finally to achroo-dextrin, which gives no reaction with iodine, and then to maltose. Prove these changes as follows:—Every minute or two take out a drop of the mixture, place it on a porcelain plate and add a drop of iodine solution. This gives first a blue color, showing the presence of starch; later a violet color, due to the mixture of the blue of the starch reaction with the red caused by the dextrin; next a reddish-brown, due to erythro-dextrin alone (starch being absent) and finally no reaction at all with iodine, proving the absence of starch and erythro-dextrin. The fluid now contains

achroo-dextrin and maltose. Test for the latter with Fehling's solution and with Barfoed's reagent.

At what stage in the process does maltose appear? Explain.

(f.) Influence of conditions on ptyalin and its amylolytic action—Report and explain the results of the following experiments:—

1. Boil a few cc. of the saliva, then add some starch paste, and place in the water-bath at 40° C. After five minutes test for sugar.

2. Take two test tubes; put some starch paste in one, and saliva in the other, and cool them to 0° C. in a freezing mixture. Mix the two solutions, and keep the mixture surrounded by ice for several minutes, then test a portion for sugar. Now place the remainder in the water-bath at 40° C. and after a time test for sugar.

3. Carefully neutralize 20 cc. of saliva with very dilute HCl (about 0.05%) and dilute the whole to 100 cc. Test the action of this neutralized saliva on starch.

4. To 5 cc. of starch paste add 10 cc. of 0.2% HCl, and 5 cc. of neutral saliva, and expose the mixture for awhile at 40° C., and test for sugar.

5. To 5 cc. of starch paste add 10 cc. of 0.5% solution of Na_2CO_3 and 5 cc. of neutral saliva, and expose the mixture for awhile at 40°C., and test for sugar.

6. Carefully neutralize (4) and (5) and again test the action of the two on starch.

(Note: By repetitions of (4) and (5) using reagents of regularly increasing degrees of dilution, the percentage of each which does not interfere with the action of ptyalin on starch can be determined. In order to obtain uniform results, a diluted saliva should be used.)

7. Mix a little uncooked starch with saliva, expose to a temperature of 40° C. for awhile, and test for sugar.

Gastric Digestion

85.—The following solutions will be found in the laboratory:—

A.—A 0.2% solution of HCl.—This is prepared by diluting 6.5 cc. of concentrated HCl (Sp. Gr., 1.19) with distilled water to one liter.

B.—A solution of pepsin.—Prepared by dissolving two grams of pepsin in 1000 cc. of water.

C.—A pepsin-hydrochloric acid solution.—Prepared by dissolving two grams of pepsin in 1000 cc. of solution A.

Or, add to 150 cc. of solution A about 10 cc. of the glycerine extract of the mucous membrane of the stomach.

(a.) Take five test tubes and label 1, 2, 3, 4, 5. Fill as indicated below. Place in a water-bath at 40° C. and examine an hour later and again the next day.

1.—3 cc. pepsin solution + 10 cc. water + a few shreds of fibrin.

2.—10 cc. 0.2% HCl + a few shreds of fibrin.

3.—3 cc. pepsin + 10 cc. 0.2% HCl and a few shreds of fibrin.

4.—3 cc. pepsin + 10 cc. 0.2% HCl, boil, cool, and then add a few shreds of fibrin.

5.—3 cc. pepsin solution + 10 cc. 0.2% HCl and a few shreds of fibrin which have been tied firmly together into a ball with a thread.

Make a note of all changes.

(b.) Filter (3). Neutralize with dilute Na_2CO_3. Filter again. Why? Test the filtrate for the Biuret reaction.

(c.) To 5 grams of fibrin add 30 cc. of the pepsin solution and 100 cc. 0.2% HCl. Set in the water-bath at 40° C., stirring frequently and leave in the water-bath over night. Observe the undigested residue on the following day and also a slight flocculent precipitate. What is this precipitate?

Filter and carefully neutralize the filtrate. A precipitate varying with the progress of the digestion will form. What is it?

Remove this by filtration and saturate this filtrate with $(NH_4)_2SO_4$. Filter. Save precipitate and filtrate. Of what does each consist?

(d.) Dissolve the precipitate of Ex. (c) in water and try the following tests:—

1.—Biuret reaction.

2.—Effect of boiling.

3.—Test with HNO_3, as in performing test for albumin in the urine.

(e.) To the last filtrate of Ex. (c) add an equal volume of 95% alcohol and stir thoroughly. The peptones will collect in a gummy mass about the stirring rod.

1.—Determine the solubility of peptones in water.

2.—What is the effect of heat when so dissolved?

3.—Try the Biuret reaction.

(f.) Demonstration of the Rennet Enzyme.—Place 10 cc. of milk in each of three test tubes. Label the test tubes 1, 2, 3.

To 1 add a drop of a neutralized glycerine extract of the mucous membrane of the stomach (made from the stomach of a calf).

To 2 add a drop of a neutralized glycerin extract and boil at once.

To 3 add a few cc. of $(NH_4)_2C_2O_4$ solution and then a drop of the glycerine extract.

Place these tubes in the water-bath at 40° C. and examine after five or ten minutes. Explain result in each case.

Continue heating tube 3 for half an hour; then add 2 to 3 drops $CaCl_2$ solution. The liquid instantly solidifies. Why?

(g). Digestion of Casein.—Determine the products of the digestion of the curd from the last experiment.

(h.) Tests for free hydrochloric acid.—Try each of the following tests with (1) HCl (0.2%, 0.05%, and 0.01% successively); (2) lactic acid (1%); (3) mixtures containing equal volumes of (1) and (2). Tabulate the results.

1.—Töpfer's Reagent.[1]—Use one or two drops of a 0.5%

[1] Organic acids and acid salts produce a somewhat similar reaction with Congo red, and with Topfer's solution, but in solutions more concentrated than those ordinarily found in the stomach contents.

alcoholic solution. In the presence of free mineral acids a carmine-red color is obtained.

2.—Gunzburg's Reagent.—Phloroglucin, 2 gms.; vanillin, 1 gm.; alcohol, 100 cc. Place two or three drops of the solution to be tested in a porcelain dish, add one or two drops of the reagent, and evaporate on a water-bath. In the presence of free hydrochloric acid a rose-red color develops.

3.—Tropaeolin OO.—Use one or two drops of a saturated alcoholic solution.

4.—Congo-Red.—Use filter paper which has been dipped into a solution of the reagent and then dried.

(i.) To 5 cc. egg albumin in solution add 1 cc. of 0.2% HCl. Mix thoroughly and test for the presence of free HCl. What is the result? How do you explain it? Repeat the test using a solution of peptone in place of the egg albumin.

(j.) Tests for Lactic Acid.—Uffelmann's reagent. Mix 10 cc. of a 4% solution of carbolic acid with 20 cc. of water, and add a drop or two of ferric chloride.

To 5 cc. of the reagent add a few drops of the lactic acid solution. Note the canary yellow color.

Does the presence of free HCl interfere with this reaction?

A more delicate reagent is obtained by adding three or four drops of a 10% ferric chloride solution to 50 cc. of water. Such a solution has a *very faint* yellow color, which is distinctly intensified by lactic acid.

Using 5 cc. of this nearly colorless solution for each experiment, note the effect of (1) 0.2% HCl, (2) di-hydrogen sodium phosphate, (3) alcohol, (4) glucose, (5) cane sugar. What conclusions do you reach concerning the value of this test, when applied directly to the gastric contents?

The test is best applied to an aqueous solution of the ethereal extract of the gastric contents:—Add to the contents two drops of HCl, boil to a syrup, and extract with ether. Dissolve the residue obtained upon evaporation of the ether in a little water, and test for lactic acid.

(k.) Quantitative Estimation of Free Hydrochloric Acid, and of Total Acidity.

1. Free Hydrochloric Acid.—To 10 cc. of unfiltered gastric juice add three to four drops of a solution of Topfer's reagent. Titrate the acidity with N/10 NaOH, taking the disappearance of the carmine red color as an end point. This point represents fairly sharply the neutralization of the free hydrochloric acid in the contents used. Acidity is generally (clinically) recorded as the number of cc. of N/10 NaOH required to neutralize 100 cc. of stomach contents. Report your analysis in this way, and also calculate the per cent of free hydrochloric acid present. Gunzburg's Reagent may also be used to determine the end point.

2.—Total Acidity.—Add two or three drops of phenolphthalein to the porcelain dish containing the stomach contents which has just been titrated for free hydrochloric acid. Continue the titration until a permanent pink color is obtained. This end point indicates the neutralization of all acid or acid salts in the stomach contents. Report the total acidity as required under free hydrochloric acid (remembering to include the free hydrochloric acid in your calculation).

Pancreatic Digestion

The experiments illustrating the action of the pancreatic juice will be performed with an extract prepared as follows:—Digest one part of finely divided pig's pancreas with four times its weight of 25% alcohol, for two or three days, and filter.

86.—(a) Proteolytic action.—To 25 cc. of a 1% solution of Na_2CO_3 add a few drops of the pancreatic extract. Place some pieces of fibrin in this liquid and keep in the water-bath at 40° C. till the fibrin has disappeared (one to two hours probably). Observe the digestion from time to time. Note that the fibrin does not swell and dissolve as in gastric digestion, but that it is eaten away from the edges.

Filter. What is the precipitate? Carefully neutralize the filtrate with 0.2% HCl. Another precipitate may appear. What is this?

Again filter, if necessary, and test the filtrate for proteoses and peptones as directed under gastric digestion.

(b.) Formation of leucin and tyrosin.—Perform a similar experiment, using boiled fibrin and adding a few drops of a 20% alcoholic solution of thymol, or a few drops of chloroform water. Why use boiled fibrin, and why add thymol or chloroform? Digest for forty-eight hours, and then examine as follows:—Filter, neutralize, and concentrate by evaporation on the water-bath. Crystals of tyrosin (and possibly leucin) usually separate. Examine microscopically.

If there is sufficient tyrosin, try Millon's test. In a portion of the residue left after separation of the tyrosin, test for tryptophan by adding, drop by drop, a very dilute solution of bromine water. The liquid becomes pale-red, then violet. To the remainder of the residue add twice its volume of 95% alcohol, which precipitates proteoses and peptones. Filter, concentrate by slow evaporation, and set aside to crystallize. Sketch the crystals of leucin and tyrosin.

(c.) Amylolytic action.—To some starch paste in a test-tube, add a drop or two of the pancreatic extract and place in the water-bath at 40° C. After a few minutes, test for sugar and report the result.

(d.) The lypolytic (fat-splitting) action.—For the demonstration of this action, refer to experiment No. 24, page 36.

URINE ANALYSIS

All urines for quantitative analysis should be collected over a definite period, usually 24 hours. Folin gives the following specific directions for this collection. He says, "The only correct way to collect 24 hour urines is to begin the metabolism period immediately after passing the night urine in the morning (i.e., immediately upon rising). Note the time and then collect all the urine passed up to the same hour the following morning.

Urines so collected must be placed in a cool and preferably dark place. Unless the entire analysis can be completed within 12 hours a preservative should be added. As these urines are not to be analyzed for glucose, the most convenient method of preserving the specimen is to add 5 cc. of a 5% solution of thymol in chloroform. Since most samples require a preservative, it is best, as a matter of routine, to place the thymol-chloroform solution in the container before any urine is added. In this way complete preservation of all material is assured.

When all urine for the period has been collected, it should be carefully measured, the specific gravity taken, the color and the reaction to litmus paper noted. If time is not available for the complete analysis of the urine at once, the estimation of uric acid, creatinine, ammonia, acidity and phosphates should be made the first day. If the specimen has been properly preserved, the remaining urinary constituents may be estimated at a later laboratory period.

In order that the student may have an idea as to the values he may reasonably expect from his analyses, the following table, taken from Folin (Am. Jour. of Physiol. XIII.) is appended.

Diet.	High Protein		Low Protein	
Volume of urine	1170 cc.		385 cc.	
Total Nitrogen	16.08 gms.		3.60 gms.	
Urea-Nitrogen	14.07	87.5%	2.20	61.7%
Ammonia-Nitrogen.	0.49	3.0%	0.42	11.3%
Uric Acid-Nitrogen.	0.18	1.1%	0.49	2.5%
Creatinin-Nitrogen.	0.53	3.6%	0.60	17.2%
Undeterm. Nitrogen	0.85	4.9%	0.27	7.3%

Total Sulph. as SO_3	3.64		0.76	
Inorganic Sulphates (SO_3)	3.27	90.0%	0.46	60.5%
Etherial Sulphates (SO_3)	0.19	5.2%	0.10	13.2%
Neutral Sulphur (SO_3)	0.18	4.8%	0.20	26.3%
Titrable acidity cc. N/10	805.0		224.0	
Mineral Acidity	389.		123.	
Organic acidity	407.		201.	
Total Phosphates, gms. P_2O_5	4.1		1.0	
Chlorine gms. Cl_2	6.1		1.6	

87. Total Nitrogen.

(a.) Kjeldahl Method:

Into an 800 cc. Kjeldahl flask
Pipette 5 cc. of Urine.
Add 15 cc. concentrated sulphuric acid
Add 2 cc. of 5% copper sulphate

Add a few glass beads or bits of glass to prevent bumping. Place on the digestion rack and digest until clear, or at most, a light straw color. Cool. Add about 400 cc. of distilled water and 2 drops of alizarine red; then prepare a receiving flask containing 25 or 30 cc. of N/10 HCl, which is placed in position below the still. Place the Kjeldahl flask on the rack. Add the calculated quantity of strong sodium hydroxide, stopper the flask, rotate to mix the caustic soda, then light the burner and distil as directed under b, page 10.

Note: To determine the proper amount of alkali required for the Kjeldahl distillation, pipette 1 cc. of concentrated sulphuric acid into a beaker containing about 25 cc. of distilled water. Add a drop of alizarin red, then add the strong caustic soda from a Mohr pipette, (noting the volume), drop by drop, stirring the beaker after the addition of each drop, until the acid is neutralized. From the amount required to neutralize 1 cc. of concentrated sulphuric acid, calculate the quantity needed for

15 cc. To this figure add 3 cc. to insure a slight excess of alkali.

Calculate the total nitrogen in the total 24 hour specimen of urine, and record this value in the table.

(b.) Folin-Farmer Method:

Preparing a urea solution so that 1 cc. contains approximately 1 mg. of nitrogen. Pipette out by means of the Ostwald pipette, 1 cc. of diluted urea solution into a large Pyrex test tube (200 x 25 mm.). Add 1 cc. of concentrated H_2SO_4, 1 gram of K_2SO_4, 1 drop of 10% $CuSO_4$ solution, and a glass bead. Boil over a micro-burner for ten minutes (in the hood). Allow the digestion mixture to cool until it just becomes viscous. (It must not solidify.) Then add about 6 cc. of water, at first adding a few drops at a time and shaking the tube gently, and then adding it more rapidly. If the tube is allowed to cool too much, the water is liable to cause the contents to solidify. In this case it is best to add the water rapidly and with vigorous shaking. In adding the water, care should be taken to wash down the sides of the test tube. Place a piece of rubber tubing about one and one-half inches long, provided with a pinch-cock, over the end of the long glass tube, which is inserted through one hole of the rubber stopper belonging to the aeration apparatus. Draw up 3 cc. of sodic hydrate into this tube, and retain by means of a pinch cock. (The tube should be marked at this point by a scratch.) Stopper the tube securely with this apparatus, taking care that the long glass tube does not project into the test tube so far as to be shoved through the bottom. Connect the apparatus to the absorption tube placed in a receiving flask. The receiving flask should contain 10 cc. of N/50 HCl, to which has been added 100 cc. of water and 3 drops of alizarine red. Care should be taken in connecting the apparatus to have all joints air tight and the absorption tube extend to the bottom of the receiving flask. Release the pinch-cock, thus allowing the alkali to flow into the test tube. Connect with the compressed air and turn on carefully, so that one or two bubbles shall mix the solutions. Run the air *slowly* for two minutes, then rapidly for fifteen minutes. At the end of this time all of the ammonia has gone over. Remove the delivery tube and

rinse it with water into the receiving flask. Titrate the excess of acid with N/50 sodic hydrate solution. Calculate from the results of analysis the nitrogen content of the urea solution, and compare with the theoretical figure. Repeat the determination upon this solution until consistent and accurate results are obtained.

Apply the above method to urine as follows:

Take the specific gravity of the urine and dilute according to the following table:

Sp. Gr.	Dilution	Take cc.	Dilute to
1.018 or less	5 times	10 cc.	50 cc.
1.018 to 1.030	10 times	10 cc.	100 cc.
1.030 or over	20 times	10 cc.	200 cc.

The dilution should be made by means of a pipette and volumetric flask. The diluted urine contains approximately 0.7 to 1.5 mg. Nitrogen per cc.

Pipette 1 cc. of this diluted urine into the large Pyrex test tube by means of the Ostwald pipette. Proceed as indicated above for the urea solution. Check your results against those obtained by the Kjeldahl method.

(c.) Total Nitrogen by Folin's Direct Nesslerization Method:

The urine is diluted as indicated in the previous method. Pipette 1 cc. of diluted urine, using an Ostwald pipette into a large Pyrex test tube (200 x 25 mm.).

Add (ordinary pipette) 2 cc. Phosphoric-Sulphuric acid digestion mixture. Add a glass bead or pebble.

Heat over a micro-burner, having the test tube only a few cm. above the top of the burner and using a low flame; until nearly all the water has been driven off. When foaming stops and dense sulphuric acid fumes appear within the test tube cover it with a small watch glass (2 to 5 min.) Regulate the flame so that only a trace of fume escapes from the tube and continue digestion until the material is clear, with only a blue, green, or light straw tint (one-half to three min. more). After this color is noted (due to the copper) continue the heating about 1 min-

ute. (The total heating time after the watch crystal has been placed in the tube should never be less than 2 minutes.)

Remove the flame and allow the test tube to cool for 2 mins. Add water and transfer quantitatively to a 100 cc. volumetric flask, using about 65 cc. of water.

Standard Solution: Pipette into a second 100 cc. volumetric flask exactly 20 cc. of Standard Ammonium Sulphate solution. This contains 1 mg. of Nitrogen. Add 40 cc. distilled water. Add 2 cc. acid digestion mixture.

Nesslerization: To each of the above flasks add 30 cc. Folin's modified Nessler Reagent. Fill to the mark with distilled water; stopper with a clean rubber stopper and mix. The Nesslerization of the Standard and Unknown should be done as nearly together as possible. A sediment may develop. This can be removed by centrifuging a portion of the sample. If the sediment is reddish in color, the determination is of no value and should be repeated.

Set the standard for 20 mm. in the colorimeter and after checking against itself (by placing some of the Standard in the cup) make the color comparison of the unknown.

Calculate the total nitrogen of the 24 hour sample of urine by the following equation:

$\frac{20}{\text{reading}}$ x dilution x cc. in 24 hr. specimen = grams of nitrogen.

Solutions Required

1. Standard Ammonium Sulphate Solution. 20 cc. = 1 milligram of nitrogen.

   Weigh out exactly 0.2358 gram of special ammonium sulphate (see Folin and Farmer, Jour. Bio. Chem. XI., 496, 1912). Dissolve in water and make to 1 liter. Preserve with chloroform.

2. Phosphoric-Sulphuric Acid Digestion Mixture.

   Measure 300 cc. H_3PO_4 (85%) into a tall cylinder. Add 100 cc. conc. H_2SO_4—set aside for precipitation of $CaSO_4$. (Settles slowly.)

Pipette off 100 cc. of clear acid mixture.

Add 10 cc. of 6% $CuSO_4$, then add 100 cc. distilled H_2O.

3. Nessler's Solution: Folin's Modification of the Nessler-Winkler Reagent.

(a.) Prepare a mercuric potassium iodide solution as follows:

Take a 500 cc. Pyrex Florence Flask;

Add 150 gms. potassium iodide, 110 gms. iodine, 100 cc. distilled water, 140-150 gms. mercury.

Shake vigorously for 7 to 15 minutes, or until dissolved iodine has nearly disappeared. Solution becomes hot. Watch the color. When red color has faded to pale red, cool in running water, shaking continuously until reddish iodine color has been replaced by greenish color of double iodide. Time of complete operation about 15 mins. Decant liquid from the mercury. Wash mercury thoroughly, with a number of changes of distilled water, adding to decanted part of liquid. Finally make decanted liquid up to 2 liters with distilled water. Final solutions should be nearly clear.

(b.) Prepare a Saturated Sodium Hydroxide Solution.

Prepare an adequate amount of completely saturated sodium hydroxide solution, which requires about 55 gms. NaOH per 100 cc. Allow to cool, then decant clear supernatant liquid. Determine strength of NaOH by titration, then calculate quantity necessary to make a 10% solution. Allowable variation 9.5% to 10.5%.

PREPARE THE NESSLER'S SOLUTION

Select a large bottle, preferably of brown glass, having a capacity of over 5 liters.

Into the bottle pour 3500 cc. of 10% sodium hydroxide solution. Add 750 cc. of the double iodide solution;

Add 750 cc. distilled water.

Mix thoroughly by shaking.

15 cc. of the above Nessler's solution contains enough alkali for the neutralization of 1 cc. of phosphoric-sulphuric acid mixture. It has also the proper alkalinity for the development of color with ammonia matching the standard solution at a volume of 50 cc.

Note: It is sometimes difficult to obtain a clear solution when Nessler's reagent is added to so concentrated a solution as that contained in the 100 cc. flask. Muddy solutions are of no value. If it is necessary a 200 cc. volumetric flask may be used, the solutions diluted to about 150 cc. before the addition of 30 cc. of Nessler's reagent.

It is imperative that the Nessler's reagent be free from suspended matter. If necessary such material may be removed by filtering the solution through the asbestos mat of a freshly prepared Gooch crucible, using suction.

88.—Determination of Ammonia Nitrogen

(a.) Folin-Macallum Areation Method:

The student should learn the technic of this method using the solution prepared as directed in No. 8, page 9. (1 cc. contains between 1-1.25 mgs. nitrogen). Consistent results checking with the theoretical should be obtained before proceeding with urine.

Procedure: Using the Ostwald pipette, measure out 2 cc. of the ammonium sulphate solution into a large Pyrex test tube. Add one-half cc. of the ammonia reagent and 2 drops of kerosene to the tube, and stopper immediately. Connect the delivery tube with the absorption apparatus and with the flask containing 10 cc. N/50 hydrochloric acid as in the total nitrogen method above. The air current should run for fifteen minutes. Remove the delivery tube, rinsing it with water and titrate as before. Compare the nitrogen content of the solution with the theoretical and with that previously obtained. Repeat your determinations until consistent and accurate results are obtained.

When the method has been learned, substitute 2 cc. of urine for the 2 cc. of ammonium sulphate solution and proceed as directed. Calculate the grams of ammonia nitrogen in the 24 hour specimen, and also its percentage of the total nitrogen.

Note: The ammonia reagent is prepared by dissolving 150 grams of potassium carbonate in water, adding to this 150 grams of potassium oxalate dissolved in water. Finally add water until the total volume is 1 liter.

The potassium oxalate is added to prevent the possibility of loss of ammonia through formation of an insoluble ammonium magnesium phosphate compound.

(b.) Permutit Method—Direct Nesslerization—Folin.

The student should check this method with 1 cc. of the ammonium sulphate solution as in the previous method before proceeding with urine.

Procedure: Weigh out 2 grams of purified Permutit on the laboratory trip scales. Place it in a 200 cc. volumetric flask. Add 5 cc. distilled water.

Add 2 cc. of urine by means of an Ostwald pipette.

Rinse urine down with a little water, 1-5 cc.

Shake gently and continuously for 5 minutes.

Rinse the permutit to the bottom of the flask with 25 to 40 cc. of water.

Decant off the water.

Add water again (25 to 40 cc.) and decant.

Repeat the above washing again if the urine contained much bile.

To the Permutit, after decanting the last water, add 2 cc. of 10% NaOH. Mix by rotating the flask, then add water until the flask is about two-thirds full. Set this flask aside until you have prepared the Standard Ammonium Sulphate solution and have diluted it to a similar volume (see below).

Shake the flask for 10 to 20 seconds, then add 20 cc. of Folin's modified Nessler reagent, gently shaking the flask as the Nessler reagent is added. Add distilled water until the flask is filled to the mark, then stopper with a clean rubber stopper, and mix thoroughly by inverting the flask several times.

Preparation of the Standard Ammonium Sulphate Solution

Pipette 20 cc. of the Standard Ammonium Sulphate solution containing 1 milligram of nitrogen into a second 200 cc. volumetric flask. A weaker standard may be prepared if necessary by taking only 15 or 10 cc. of the above solution.

Add 2 cc. of 10% NaOH.

Add distilled water until the flask is two-thirds full.

Add 20 cc. of modified Nessler's reagent, as directed above.

Dilute with water to the mark, stopper flask and mix contents.

Note: It is important that the standard and unknown solutions be nesslerized as nearly together as possible.

Compare the solutions in the colorimeter, setting the standard at 20 mm., as directed under total nitrogen.

Calculate the amount of ammonia nitrogen in grams in the 24 hour specimen, and also the percentage of total nitrogen.

Preparation of a Purified Permutit: Permutit is the trade name for an artificial zeolite of complex chemical nature. It may be looked upon as a sodium aluminum silicate, in which the sodium is easily exchanged for ammonia or vice versa, depending upon their respective concentrations.

In ordering Permutit indicate it as follows, "Permutit prepared according to Folin" to secure the proper grade. Before being used it is washed once with 2% acetic acid and twice with distilled water. If desired, this may be done in the volumetric flask before the addition of the urine.

Permutit may be used repeatedly if washed free from alkali and treated with 2% acetic acid as above. The purified material should be air dried, as oven temperature decreases its efficiency.

89.—Urea Nitrogen. Folin's Application of the Urease Method. Areation and Direct Nesslerization.

The student should learn the technic of this method using a urea solution of known concentration. If 2 to 3 grams of urea be accurately weighed out, dissolved in water and made to a volume of 1 liter; 1 cc. of that solution will contain a little more than 1 milligram of urea nitrogen. To facilitate the class work, a solution so prepared may be obtained from the stock room. Caution: This solution requires no further dilution as in urine.

Procedure: Dilute the urine according to its specific gravity as directed under section b, total nitrogen above.

Pipette 1 cc. of urea solution or diluted urine into a large Pyrex test tube. (This test tube should previously be rinsed with nitric acid, to remove any trace of mercury; then well washed with distilled water.)

Add 1 to 2 drops (not more) of phosphate buffer.

Add 1 cc. of urease solution.

Place in a beaker of warm water (40° C. to 55° C. *as shown by the thermometer*) for 10 to 15 minutes.

The procedure to this point is the same irrespective of the mean by which the ammonia formed by urease digestion is to be evaluated. Complete the determination by either of the following methods.

a.—Areation and Titration

Pipette 10 cc. N/50 hydrochloric acid into a 250 cc. Florence flask. Add 2 drops of alizarine red. Use this to receive the ammonia, as indicated in the areation method under total nitrogen.

To the large test tube containing the dilute urine and urease,

Add 2 drops kerosene.

Draw 2 cc. 10% NaOH up into the air tube of the areation apparatus, holding it there by the short piece of gum tubing and pinch cock fitted over the end (see total nitrogen method).

Stopper the test tube.

Connect to the perforated tube in the receiving flask.

Complete the procedure and back titrate with N/50 NaOH as directed under the Folin-Farmer method for total nitrogen.

From your results calculate the number of grams of urea nitrogen in the 24-hour specimen. Remembering that this method gives not only the urea nitrogen, but also the ammonia nitrogen; subtract the ammonia nitrogen as determined under No. 88 above, the remainder then equals the grams of urea nitrogen per 24 hours. Calculate also in percentage of the total nitrogen.

b.—Direct Nesslerization

At the end of the 15-minutes digestion period, transfer the contents of the large Pyrex tube to a 200 cc. volumetric flask, rinsing the tube with distilled water and then adding water until the flask contains about 150 cc. of fluid. Prepare the standard solution as follows:

Pipette 20 cc. standard ammonium sulphate solution (containing 1 milligram of nitrogen) into a second 200 cc. volumetric flask.

Add 1 cc. of urease solution.

Add 1 to 2 drops of phosphate buffer.

Add water to a volume of 150 cc.

To each flask (standard and unknown) add 20 cc. of Nessler's reagent. (It is sufficiently accurate to measure out Nessler's reagent with a cylinder). The flasks should be gently rotated while the Nessler's reagent is being added. To each, add distilled water to the 200 cc. mark. Stopper, and invert to thoroughly mix the solution.

Compare the solutions in the colorimeter, setting the standard at 20 mm. From this calculate the combined urea and ammonia nitrogen in the 24-hour specimen. Subtract the ammonia nitrogen, the difference being the urea nitrogen.

Also calculate the percentage of urea nitrogen to the total nitrogen.

Two solutions require preparation:

1. Jack Bean Urease Solution:

Weigh out 3 grams of Permutit on the trip scale. Place it in a 250 cc. Florence flask. Wash once with

2% acetic acid, then twice with distilled water. Add 5 grams of Jack Bean meal, then 100 cc. of 15% alcohol. Shake gently and continuously for 15 minutes. Now place a fluted filter paper in a large funnel. Pour the material from the flask into the funnel, then cover with a watch crystal, collecting the filtrate in a number of small bottles. These bottles should be stoppered, then placed in the ice box. When required, one bottle may be opened and depended upon to retain its strength for 1 week. The unopened bottles retain their activity for 6 weeks, when kept in the refrigerator.

2. Phosphate Buffer Solution:

Folin recommends two phosphate buffer solutions, one a mixture of the mono- and di-sodium phosphates (1:2) and the second, a solution of 14 grams of sodium pyrophosphate ($Na_4P_2O_7 \cdot 10\ H_2O$) in sufficient half normal ortho-phosphoric acid to make 100 cc.

For work with classes in this laboratory, we prefer the first mentioned buffer mixture. It is easily prepared, keeps unchanged and gives excellent results. To prepare it, dissolve 69 grams of monosodium phosphate ($NaH_2PO_4 \cdot H_2O$) in warm distilled water, add 179 grams of crystallized disodium phosphate ($Na_2HPO_4 \cdot 12H_2O$) and sufficient water to get it into solution, finally making the volume to 1000 cc. when cold.

90.—Uric Acid

a. Preparation from Urine, and Qualitative Tests.

1. Preparation of Uric Acid from Urine.

Place 500 cc. of urine in a large beaker, and add 25 grams of ammonium sulphate. Stir until the ammonium sulphate is completely dissolved. Add 10 cc. of strong ammonia and allow to stand over night. Crystals of ammonium urate should form. Filter these off, and wash two or three times with a little water, containing some ammonia. Place the crystals in a small beaker containing a few cc. of hydrochloric acid, and allow it to stand until the next day. Examine the crystals under the microscope.

2. Reactions of Uric Acid. Murexide Test.

Place a few crystals of uric acid in a small porcelain evaporating dish. Add 2 drops of concentrated nitric acid, and evaporate cautiously to dryness on the water bath, or over a very low flame. A red, or yellowish-red color appears. After the residue is cooled, add a drop of very dilute ammonium hydroxide solution. A purplish-red color should appear. The development of color is due to the formation of a substances called murexide. A purplish-violet color is obtained if potassium hydrate is used instead of ammonium hydrate. This is due to the formation of the potassium salt of murexide. Repeat the test, using caffein instead of uric acid. (Note: The color developed by means of potassium hydrate disappears quickly on warming with uric acid, thus distinguishing it from purines.)

3. Folin-Dennis Reaction.

Dissolve a few crystals of uric acid in 2 cc. of very dilute sodic hydrate solution. Add 1 cc. of the uric acid reagent (to be described later), and then 10 cc. of saturated sodium carbonate solution. A pronounced blue color should be obtained.

b. Quantitative Determination of Uric Acid. Titration. Folin-Shaffer Method.

1. Solutions Required:

One liter of N/20 potassium permanganate solution (1.5780 gms. of $KMnO_4$ per liter). The Folin-Shaffer reagent, consisting of 500 grams of ammonium sulphate, 5 grams of uranium acetate, and 60 cc. of 10% acetic acid in 560 cc. of distilled water. A uric acid solution prepared by dissolving 0.5 to 0.6 grams of uric acid in 600 cc. of water, to which has been added 1 gram of lithium carbonate. The solution is then diluted to one liter.

2. Checking of Solutions:

The above solutions should be checked against each other in the following manner: 100 cc. of the

uric acid solution is pipetted out into a beaker. Twenty cubic centimeters of concentrated sulphuric acid are added to the solution, which is titrated immediately with N/20 potassium permanganate solution. The first permanent pink coloration should be taken as an end point. Each cubic centimeter of potassium permanganate equals 3.76 mgs. of uric acid.

3. Preliminary Experiment:

Measure out two samples of 100 cc. of the uric acid solution into a beaker. Add 10 grams of solid ammonium sulphate and stir until dissolved. Then add 5 cc. of concentrated ammonia. Cover the beaker with a watch crystal, and allow it to stand for forty-eight hours. Filter off the precipitated ammonium urate. Open the filter paper and wash the precipitate back into the beaker by means of a very fine jet of water. Not *more or less* than 100 cc. of water should be used in this operation. Add 20 cc. of concentrated sulphuric acid, and titrate immediately as described under 2. The results of the two determinations should agree.

4. Determination of Uric Acid in Urine:

Pipette out 150 cc. of fresh urine into each of two beakers. Add exactly 37.50 cc. of the Folin-Shaffer reagent.* Stir vigorously. Allow the solution to stand for a few minutes until it settles, and then decant off the clear liquid. If the solution does not settle readily, it should be filtered. Measure out 125 cc. of the filtrate (which corresponds to 100 cc. of urine) into a beaker. Add exactly 5 cc. of concentrated ammonia, and after covering the beaker with a watch crytsal, allow it to stand for forty-eight hours. At the end of this time, filter off the ammonium urate onto a small filter paper. Wash the precipitate five or six times with small portions of 10% ammonium sulphate solution. Wash the precipitate back into the beaker, and finish the determination, as described above. Calculate the 24-hour quantity of

uric acid and of uric acid nitrogen in the specimen of urine.

*This reagent precipitates the phosphates in the urine. Substances of a mucoid nature, which are present, are removed during this precipitation. The object of this precipitation is to remove those substances other than uric acid, which may be oxidized by potassium permanganate. The ammonium sulphate serves to decrease the solubility of the ammonium urate, which is formed later in the procedure by the addition of ammonia.

c. Colorimetric—Determination of Uric Acid in Urine. Benedict-Hitchcock Modification of the Folin-Macallum-Denis Procedure.

This method gives trustworthy results with an amount of uric acid ranging between 0.7 and 1.3 milligrams. Ordinarily 2 cc. of urine is sufficient to obtain the required quantity of uric acid, but with very dilute urines, 3 cc. or even 4 cc. may be required.

Procedure: Pipette 2-4 cc. urine (Ostwald pipette) into a 15 cc. centrifuge tube.

Add water to a volume of 5 cc.

Add 15-20 drops ammoniacal silver mangesium mixture.

Mix thoroughly by stirring with small glass rod.

Centrifuge 1 to 2 minutes.

Decant all possible fluid.

Drain standing on filter paper for two minutes.

Add 2 drops of 5% potassium cyanide.

Stir with small glass rod previously used for ½ minute, or until the precipitated silver urate is dissolved.

Add 10 to 15 drops water.

Stir thoroughly. Any precipitate remaining is probably magnesium ammonium phosphate. It will do no harm.

Add 2 cc. uric acid reagent.

Stir thoroughly.

Add 10 cc. of 20% sodium carbonate (anhydrous).

Transfer to a 50 cc. volumetric flask.

Allow to stand ½ minute. Dilute to 50 cc. with distilled water.

Compare in the colorimeter with a standard, prepared simultaneously, in the following manner:

Uric Acid Standard:

Pipette 5 cc. standard uric acid solution (1 milligram uric acid) into a 50 cc. volumetric flask.

Add 2 drops of 5% potassium cyanide.

Add 2 cc. uric acid reagent.

Add 10 cc. of 20% sodium carbonate.

Allow to stand ½ minute. Dilute to 50 cc. volume with distilled water.

Set the standard at 15 mm.

Calculation: $\frac{15}{\text{unknown}}$ = mgs. uric acid in quantity of urine taken. From this calculate the grams of uric acid, and uric acid nitrogen per 24 hours. Also calculate the percentage of total nitrogen.

Solutions Required:

1. Ammoniacal Silver Magnesium Mixture:
   - 3% silver lactate solution..... 70 cc.
   - Magnesia mixture ..... 30 cc.
   - Concentrated ammonium hydroxide..... 100 cc.

2. Magnesia Mixture (used above):
   Dissolve—
   - 175 gms. magnesium sulphate
   - 350 gms. ammonium chloride

   in 1400 cc. distilled water.
   Add 700 grams concentrated ammonium hydroxide.

3. 5% Potassium Cyanide. Label bottle "Poison"—Handle with Care.

4. Modified Uric Acid Reagent.
   Place the following material in a 1½-liter Pyrex flask:
   - 100 grams of sodium tungstate.
   - 30 cc. of orthophosphoric acid (85%).
   - 20 cc. of concentrated hydrochloric acid.
   - 750 cc. of distilled water.

   Boil for 1½ hours, using a reflux condenser. Cool, make up to 1 liter with distilled water.

5. A 20% solution of sodium carbonate. The anhydrous salt must be used.

6. Standard Uric Acid Solution. Dissolve together:
   9 grams pure dibasic sodium phosphate (Na_2HPO_4-$12H_2O$)
   1 gram pure monobasic sodium phosphate (NaH_2PO_4-H_2O)
   in 300 cc. hot distilled water.
   Filter if not perfectly clear.
   Add hot distilled water to 500 cc. volume.

   When filtered, pour this hot solution upon *exactly 200 milligrams* of pure uric acid, suspended in 5 to 10 cc. of distilled water, in a 1-liter flask. Shake gently until the uric acid is completely dissolved. Cool, add *exactly 1.4 cc.* of glacial acetic acid, dilute to 1 liter with distilled water, stopper and mix. Add 5 cc. of chloroform to prevent formation of moulds. Five cubic centimeters of this solution contains exactly 1 milligram of uric acid. When properly preserved, in a well-stoppered bottle, this solution shows no change upon keeping.

d.—Benedict-Franke Direct Colorimetric Method for Uric Acid.

This method is the latest and simplest reliable colorimetric method for uric acid in urine. If the urine contains albumin, it must be removed by slightly acidifying with acetic acid, coagulated by boiling, and removed by filtration.

Procedure: Dilute the urine so that 10 cc. will contain between 0.15-0.30 mg. uric acid. (Usually 5 cc. of urine diluted to 100 cc. is satisfactory.)

Pipette 10 cc. diluted urine into a 50 cc. volumetric flask.
Add 5 cc. of 5% sodium cyanide solution *from a burette.*
Add 1 cc. of arseno-phosphotungstic acid reagent.
Mix, by shaking; allow to stand 5 minutes and then dilute to 50 cc. with distilled water.

Prepare at the same time the following standard so that the color in the two solutions will be developing simultaneously.

Standard Solution: Pipette 10 cc. standard uric acid solution into a 50 cc. volumetric flask.

Add 5 cc. of 5% sodium cyanide solution *from the burette.*

Add 1 cc. of arseno-phosphotungstic acid reagent.

Mix by shaking; allow to stand 5 minutes, then dilute to 50 cc. volume as indicated above.

Compare the solutions in the colorimeter, setting the standard at 15 mm.

Calculation: $\frac{15}{R} \times 0.2 =$ milligrams of uric acid in 10 cc. of the *diluted* urine used.

Calculate the grams of uric acid and of uric acid nitrogen in the 24-hour sample. Also calculate the percentage of uric acid nitrogen to the total nitrogen.

Solutions:

1. Arseno-phosphotungstic Acid Reagent. Put 100 grams of sodium tungstate (C. P.) into a 1000 cc. Pyrex flask. Add 600 cc. of water and when dissolved add 50 grams of pure arsenic acid (P_2O_5). Add 25 cc. of 85% ortho phosphoric acid and 20 cc. of concentrated hydrochloric acid. Boil for 20 minutes, cool, and dilute to 1000 cc.

2. A 5% solution of sodium cyanide. This should be prepared fresh each week.

3. Standard Uric Acid Solution . Ten cubic centimeters of this solution contains 0.2 mg. of uric acid. To prepare it, pipette 50 cc. of the phosphate standard solution given on page 123, No. 6, into a 500 cc. volumetric flask. Add distilled water to a volume of 400 cc., then add 25 cc. of dilute hydrochloric acid (1 part HCl plus 9 parts of distilled water), and dilute to 500 cc. with distilled water. Mix. Prepare a fresh standard each week.

91.—Creatinin

a. Jaffe Test. Place 100 cc. of urine in a beaker and add to it 20 cc. of a 5% alcoholic solution of picric acid. The precipitate is a picrate of creatinin. Allow the solution to stand for one-half hour, then filter off the crystals and wash with a very little cold water. Dissolve a part of the precipitate in dilute sodium hydrate solution. Slightly acidify the mixture, and notice the change in color. Repeat the experiment with 10 cc. of urine,

adding from 3 to 5 cc. of a saturated aqueous solution of picric acid and 1 cc. of a 10% sodium hydroxide solution. The intensity of the color is dependent upon the amount of creatinin present. Folin makes use of this test for his quantitative estimation of creatinin in urine.[1]

b. Determination of Creatinin in Urine.

1. Folin's Original Method. Place 10 cc. of urine in a 500 cc. volumetric flask. Add 15 cc. of a saturated aqueous solution of picric acid and 5 cc. of 10% sodium hydroxide solution. Mix the liquids thoroughly, and allow them to stand for exactly five minutes. During this interval pour a little N/2 potassium bichromate solution into each of the two cylinders of the Dubosq colorimeter. Adjust the depth of the liquid in the left-hand cylinder to 8 millimeters. Make a few preliminary readings by comparing the color in the two halves of the field. The two readings should be identical. At the end of five minutes, fill the flask up to the 500 cc. mark with tap water. Shake the solution so that it is thoroughly mixed. Read immediately by replacing the potassium bichromate solution in the right-hand cell with this creatinin solution. If the creatinin content of the urine is such that a reading of over 15 or under 5 millimeters is obtained, repeat the determination, varying the amount of the urine taken, so that the color of the solution may approximate the standard more closely.

When 10 milligrams of pure creatinin are put into solution, and the color developed as stated for urine, exactly 8.1 millimeters of this solution balance 8 millimeters of an N/2 potassium bichromate solution. The potassium bichromate solution is permanent in color, whereas the color of the creatinin potassium picrate fades rapidly. Determine the amount of creatinin in the volume of urine taken by the following equation:

$$a : 8.1 :: 10 : x$$

where a is the observed reading in millimeters, x is the milligrams of creatinin in the volume of urine taken. The proportion is inverse on account of the fact that the instrument is so constructed that the greater the color, the lower the numerical

[1] A very interesting paper dealing with the nature of the reaction between picric acid and creatinin in a cold alkaline solution was published by Chapman. See "On Jaffe's Colorimetric Method for the Estimation of Creatinine." A. C. Chapman, The Analist, XXIV., 475, 1909.

value of the reading. The creatinin equivalent of the potassium bichromate is substituted, i. e., 8.1 millimeters. Calculate the 24-hour quantity of creatinin and also of creatinin per kilogram and per pound of your body weight.

2. Folin's New Method. Pipette (Ostwald pipette) 1 cc. to 2 cc. urine into a 100 cc. volumetric flask. Mark this *urine*.

Pipette (Ostwald) 1 cc. of standard creatinin solution (containing 1 mg. creatinin) into a second 100 cc. volumetric flask. Mark this *standard*.

To each flask:

Add 20 cc. of saturated picric acid solution.

Add (Mohr pipette) 1.5 cc. 10% sodium hydroxide solution.

Allow both flasks to stand for 10 minutes.

Dilute at the end of 10 minutes to 100 cc. volume.

Stopper and mix and compare in the colorimeter, setting the standard at 20 mm.

Calculation: $\frac{20}{\text{unknown}}$ = milligrams of creatinin in the quantity of urine taken.

Calculate the total amount of creatinin in the 24-hour sample of urine. Also calculate the percentage of creatinin nitrogen. Determine the number of milligrams of creatinin per kilogram of body weight.

Solutions Required: 1. The picric acid used for the above creatinin estimations must be carefully purified according to the method of Folin and Doisy (Jour. Biol. Chem. 28-349, 1916-17). From the purified picric acid, as a supply a saturated aqueous picric acid should be prepared.

2. Standard creatinin solution may be most conveniently made by weighing out 1.61 grams of creatinin zinc chloride, and dissolving it in 1 liter of N/10 hydrochloric acid. One cubic centimeter of this solution contains 1 milligram of creatinin.

92.—Creatin

Folin has worked out a method whereby the creatin occurring in the urines of children and adults suffering from severe tissue destruction, as in fevers, may be transformed to creatinin,

and estimated along with the pre-formed creatinin. It should be remembered that creatin gives no color with an alkaline solution of picric acid. Normal adult individuals have no creatin in their urines, unless eating large amounts of fleshy foods, in which it exists preformed.

Folin's Procedure (Creatinin plus Creatin).

Pippette (Ostwald pipette) 1 cc. urine into a 300 cc. Florence flask.

Add 20 cc. saturated picric acid solution.

Weigh the flask so prepared on the laboratory trip scale.

Add 150 cc. water.

Boil *gently* for 45 minutes.

Weigh again, and unless this weight is within 3 or 4 grams of the original, return to the flame and boil rapidly to its original weight.

Cool to room temperature.

Add 1.5 cc. 10% sodium hydroxide.

Allow to stand 10 minutes.

Transfer at the end of 10 minutes to a 100 cc. volumetric flask.

Add distilled water to the mark.

Stopper—shake, etc.

Compare in the colorimeter with the standard described above under Creatinin. The standard should, of course, be freshly made up, and the color developed simultaneously in both standard and unknown.

From the value obtained, subtract the value for the pre-formed creatinin, the difference is the creatin. Calculate the total amount of creatin in the 24-hour specimen of urine.

Folin gives the following shorter method which may be used if an autoclave is available.

Pipette (Ostwald) 1 cc. of urine into a 100 cc. volumetric flask.

Add 20 cc. saturated picric acid solution.

Cover mouth of flask with tin foil.

Heat in autoclave at 115°-120° C. for 20 minutes.

Cool to room temperature.

Add 1.5 cc. sodium hydroxide. Proceed as above.

93.—Inorganic Sulphates—Folin Method

a. The student must now learn to use the Gooch crucible. This crucible, when fitted with a proper mat of asbestos, is used in place of filter paper to collect the precipitated barium sulphate. Furthermore, the subsequent process of ignition and weighing of precipitates are most easily accomplished.

In order to be of most service, the Gooch crucible must be provided with an asbestos mat which will permit rapid filtration with moderate vacuum, and yet retain the finest particles of precipitate.

To form the asbestos mat, proceed as follows: Shake thoroughly the stock bottle of "asbestos soup," then pour about 50 cc. into a small beaker. Place the Gooch crucible in the rubber holder, disconnecting the rubber tubing from the suction flask. Agitate the asbestos soup in the beaker, then pour about 15 to 20 cc. into the crucible, allowing it to drain completely. Now apply very gentle suction, causing the fibres to "felt" together without plugging the holes in the crucible. This should give a firm and uniform felt of 1/32 inch thickness. Wash the mat thoroughly with distilled water while the suction is turned on, using about 500 cc. This should remove all loose fibres. Stop suction, disconnect rubber tubing, remove crucible, empty flask, rinse flask with distilled water, then return crucible to holder. Open suction cock for medium suction and connect tubing to flask. Now pour 100 to 200 cc. more distilled water through crucible, examining the filtrate for loose asbestos fibres. If none are visible in filtrate when poured into beaker for examination, the mat is in condition for drying.

Place the crucible in a small, clean beaker, labeled with your name, and put into the drying oven (or steam oven) for 20 minutes. At the end of this time, remove the crucible and ignite over medium Bunsen flame, while resting on a porcelain crucible cover, supported by a clay triangle, on an iron tripod.

After the crucible has been ignited for ten minutes, transfer with clean crucible tongs to the dessicator. Allow to cool thoroughly (45 min. at least), weigh, and record weight. Re-ignite, cool in dessicator, and re-weigh. Repeat until weights are constant within 0.0003 gm.

Always handle prepared crucible with clean tongs. When crucible is to be used for filtration, always turn on gentle suction before fitting into rubber adapter on funnel. Never flood felt in bottom of crucible with strong current of water or solution, but pour down side of crucible, using stirring rod as guide, *with suction turned on.* Always test crucible before using, for leaks in mat by running 100 cc. distilled water through it, examining filtrate for asbestos fibre. If leaks are discovered, discard, and remake mat. *Two crucibles should be prepared in the above manner.*

b. The student should run a sulphate determination upon an ammonium sulphate solution of known strength.

Procedure: Pipette into a small beaker 25 cc. of the ammonium sulphate solution prepared as directed on page 8, No. 8. Dilute with water to about 100 cc. Add 10 cc. of a 20% solution of sodium chloride (free from sulphates). Acidify by the addition of 5 cc. of concentrated hydrochloric acid. Add carefully, drop by drop and without shaking, 15 cc. of 5% barium chloride solution. Allow to stand quietly for ½ hour, then stir to completely precipitate all the sulphate. After 1 hour, filter the precipitated barium sulphate into the Gooch crucible, and wash with distilled water until the filtrate no longer gives a test for chlorides. Dry the crucible and precipitate, ignite, cool in the dessicator and weigh. From the increase in weight, due to the barium sulphate, calculate the amount of sulphur (as SO_3) in the 25 cc. of ammonium sulphate solution. Compare with the theoretical figure.

Repeat the above, using duplicate determinations on 25 cc. of urine. From the determinations calculate the amount of inorganic sulphate (in grams of SO_3) in the total 24-hour sample. Also later, calculate the per cent of inorganic sulphates to the total sulphur (as SO_3).

Note: Folin claims that the addition of 10 cc. of sodium chloride to the urine is unnecessary. He also states that no hydrolysis of ethereal sulphates occurs even if the precipitated barium sulphate is not filtered off at the end of 1 hour.

94.—Total Sulphates—Folin Method

Pipette out 25 cc. of urine into a beaker, and add 5 cc. of

concentrated hydrochloric acid. Cover the beaker with a watch crystal and boil very gently for fifteen minutes. Cool; add sufficient water to make the volume 100 cc., then add barium chloride and proceed as in the above determination, No. 93.

Note: The ethereal sulphates are decomposed when boiled with the hydrochloric acid.

Calculate the total sulphates as SO_3 in the 24-hour urine. Later calculate the percentage to total sulphur in terms of SO_3.

95.—Ethereal Sulphates

Procedure No. 93 gives the inorganic sulphate content, while procedure No. 94 gives the total sulphates occurring in urine. The latter contains both the inorganic and the ethereal sulphates. It is evident, therefore, that by subtracting the value obtained for the inorganic sulphates from that obtained for total sulphates, the remainder will represent the ethereal sulphates present in urine. Figure this quantity in terms of SO_3 in the 24-hour amount of urine. Later calculate its percentage of total sulphur (as SO_3).

96.—Total Sulphur — Denis' Modification of Benedict's Method

Pipette 25 cc. of urine into a 300 cc. Pyrex beaker. Add exactly 5 cc. of the oxidizing reagent. Evaporate the mixture to dryness on the water bath. Place in the hot-air oven, and dry at 110° C. for one-half hour. Gently heat the beaker over the flame of a Fletcher burner until the organic matter is burned off, then gradually increase the temperature until the beaker is at a dull red heat, and continue at this temperature for ten to fifteen minutes. Allow to cool.

Add 20 cc. of dilute hydrochloric acid (1-4), and warm gently until the residue goes completely into solution. Add 100 cc. of hot water. Heat the solution to boiling and add, drop by drop, 25 cc. of 10% barium chloride solution. Filter, wash free from chlorides, dry in the oven, and ignite as described above. Calculate the total sulphur in terms of sulphur and SO_3 in the 24-hour amount of urine.

(Note: Vigorous oxidation is necessary to decompose certain organic sulphur-containing substances in urine. These bodies are decomposed, and their sulphur oxidized to sulphate by the

reagent. The value of the so-called "neutral sulphur" is obtained by subtracting the value obtained in the determination of total sulphates from the value obtained in this determination (both being figured as S or SO_3).

All sulphur determinations must be run in duplicate. Solution: The oxidizing reagent is made by dissolving 25 gms. of copper nitrate, 25 grams of sodium chloride (free from sulphate) and 10 grams of ammonium nitrate in water, making the final volume to 100 cc.

97.—Total Phosphates. Quantitative Estimation by the Uranium Acetate Method

Place 50 cc. of urine in a small beaker or a small Erlenmeyer flask, and add 5 cc. of a special sodium acetate solution. Heat the mixture to boiling. Run into the hot mixture from the burette a standardized solution of uranium acetate. The mixture should be added in small portions, boiling after each addition, and stirred with a glass rod. The faintest permanent brownish-red color, which is produced when a drop of the mixture is touched against *a few crystals* of powdered potassium ferrocyanide on a tile, should be taken as the end point. Caution: Rinse rod off before returning to beaker.

Repeat the determination with another specimen of urine, and calculate the total phosphate content of the 24-hour specimen.

The values should be recorded both in grams of phosphorus pentoxide (P_2O_5), and as cubic centimeters of tenth-normal acidity (7.1 milligrams of P_2O_5= 1 cc. N/10 acidity).

Solutions: The sodium acetate solution is prepared by dissolving 100 grams of sodium acetate in 800 cc. of distilled water. To this, 100 cc. of 30% acetic acid is added, and the mixture made up to 1 liter.

The uranium acetate solution is prepared by dissolving about 34 grams of uranium acetate in water. Fifty cubic centimeters of strong acetic acid is added. After the salt is in solution, the volume is made up to 1 liter with distilled water. The solution should be filtered if not perfectly clear.

The solution is standardized against a monopotassium phos-

phate solution, prepared by weighing out exactly 1.3102 grams of recrystallized potassium dihydrogen phosphate (KH_2PO_4), dissolving it in water, and diluting to a volume of 250 cc. Fifty cubic centimeters of this solution contain 0.1 gram of phosphorus pentoxide. Fifty cubic centimeters of the phosphate solution are pipetted out into a beaker and 5 cc. of a special sodium acetate solution are added. This is heated to boiling, and the uranium solution carefully run in until no further precipitation of phosphate is noticed. Continue adding the uranium solution until a drop of the mixture, when removed and touched against a *few crystals* of powdered potassium ferrocyanide, produces a faint brownish-red color. The quantity of uranium acetate solution required to titrate the phosphate mixture is equivalent to 0.1 gm. of phosphorus pentoxide (P_2O_5). The value of 1 cc. of the uranium solution is obtained by dividing 0.1 gm. (the value of the phosphate solution) by the number of cubic centimeters of uranium acetate solution used. This value, when determined, should be recorded on the label of the bottle.

98.—Acidity of Urine—Quantitative Estimation of the Acidity of Urine—Folin Method.

Titrate the acidity of 25 cc. of urine, to which has been added 5 cc. of a saturated solution of potassium oxalate. Phenolphthalein should be used as an indicator. Titrate the acidity of 25 cc. of urine without the addition of potassium oxalate and compare with the value obtained in the above titration. The disturbing influence of calcium salts forming with the phosphates present a tri-basic phosphate as the alkali is added, is prevented by the addition of the potassium oxalate. The effect of the ammonia upon phenolphthalein is also somewhat decreased by the addition of this salt. Calculate the total acidity in a 24-hour specimen of urine in terms of cubic centimeters of N/10 acid. From these figures, what do you conclude as to the condition of phosphates in urine?

99.—Chlorides—Volhard Method

Pipette out 10 cc. of urine into a 100 cc. volumetric flask. Add 50 cc. distilled water, 5 cc. of a 5% ammonium iron alum solution, and 5 cc. of concentrated nitric acid. Add 20 cc. of standard silver nitrate solution by means of a pipette. Fill up to the mark with distilled water, and shake. Filter the mixture,

and pipette our 50 cc. of the filtrate. Titrate this with a standard ammonium sulphocyanate solution. The amount of sulphocyanate solution used, when multiplied by 2, gives at once the excess of silver nitrate. The difference between this and 20 represents the quantity required to precipitate all of the chlorides in 10 cc. of urine. Calculate the number of milligrams of sodium chloride in the 24-hour specimen of urine.

Solutions:

Silver nitrate solution contains 7.2650 grams of purest obtainable silver nitrate dissolved in water and made up to 250 cc. volume. One cubic centimeter of this solution equals 10 milligrams of sodium chloride.

The ammonium sulphocyanate solution is made by dissolving 3.25 gms. of the salt in water and making up to 250 cc. volume. One cubic centimeter of this solution should be exactly equivalent to 1 cc. of the silver nitrate solution. The solutions should be titrated against each other to determine if this ratio is correct.

100.—Indican—Folin Method

Put 10 cc. of urine in a long test tube, and add 2 cc. of a 5% copper sulphate solution and 5 cc. of chloroform. Add to this mixture 17 cc. of concentrated hydrochloric acid. Place the thumb across the mouth of the test tube, and shake vigorously for one-half minute. A blue color, sometimes tinged with red, appears in the chloroform. This is due to the presence of indican in the urine, and the development of color is proportional to its amount. This qualitative test is frequently made roughly quantitative by using the color of Fehling's solution as a standard, calling it 100.

BONE[1]

101.—A. *Gelatin.*—Gelatin is prepared as described on page 87.

B. Mineral Constituents:

1. Test for phosphoric acid as follows: To a portion of the hydrochloric acid solution from Experiment 81-D-2-A (p. 87), add nitric acid, then twice its volume of a solution of ammonium-molybdate, and warm *gently*. A yellow precipitate of ammonium-phosphomolybdate should form.

2. Treat some bits of bone with dilute nitric acid, and test a small portion for chlorides by the addition of silver nitrate.

3. Test for calcium and magnesium as follows: Add to a portion of the hydrochloric acid solution an excess of ammonia. Calcium phosphate and magnesium phosphate are thrown down as a white precipitate. Filter off the precipitate, and to the filtrate add ammonium oxalate. A white precipitate shows the presence of calcium, not in the form of a phosphate. Wash the precipitate upon the filter paper and dissolve in dilute hydrochloric acid. To this solution add ferric chloride, drop by drop, until a drop of the solution gives a yellowish precipitate when mixed with a drop of ammonium hydrate. Add sodium carbonate until nearly neutralized, and then barium carbonate to precipitate ferric phosphate. Filter, heat the filtrate, and precipitate the barium by adding just the right amount of dilute sulphuric acid. Filter off the barium sulphate. The filtrate contains calcium and magnesium phosphates. The calcium phosphate is precipitated after making the solution alkaline with ammonia by the addition of ammonium oxalate. This salt should be added slowly and as long as a precipitate of calcium oxalate forms. Filter off the precipitate, and add to the filtrate sodium phosphate, which, in the ammoniacal solution, precipitates magnesium as a white ammonium magnesium phosphate.

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[1] Consult Mathew's Chapter XV.

MILK[1]

102.—Determine the specific gravity of milk.

Proteins of Milk

103.—Pipette out 5 cc. of milk into a Kjeldahl flask, and add 20 cc. of sulphuric acid, 10 grams of potassium sulphate, and 1 cc. of 5% copper sulphate. Heat gently until the charred mass ceases to froth. Then increase the heat and continue the digestion for about two hours, or until all of the carbonaceous matter has been oxidized. From this point, continue the determination as described under total nitrogen, Experiment No. 9. Knowing the specific gravity and the volume of the milk used, determine its nitrogen content. Also figure this result in terms of protein by multiplying the nitrogen content by the factor 6.25.

Caseinogen

104—Mix about 20 grams of milk with 40 cc. of a saturated solution of magnesium sulphate, then add the salt in substance until no more will dissolve. The precipitate consists of caseinogen admixed with a little fat and lacto-globulin. Filter off the precipitate, wash it thoroughly with a saturated solution of magnesium sulphate (preserving the filtrate and washings for the determination of lactalbumin). Transfer the filter and precipitate to a Kjeldahl flask, and determine the nitrogen content according to the directions given in the previous experiment. Calculation: Multiply the total nitrogen by the factor 6.25 to obtain the amount of casein. Determine also the percentage of casein nitrogen to the total nitrogen.

Lactalbumin

105.—To the filtrate and washings from the determination of caseinogen add Almén's[2] reagent until no more precipitate forms. Filter off the precipitate and determine the nitrogen content according to the method given under protein. Multiply the total nitrogen by the factor 6.25 to obtain the amount of lactalbumin.

Lactose

106.—Lactose is prepared in Experiment 50, page 56.

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[1] See Mathew's p. 306.

[2] Almén's reagent may be prepared by dissolving 5 grams of tannin with 240 cc. of 50% alcohol and adding 10 cc. of 25% acetic acid.

Determination of Milk Sugar

107.—Pipette out 50 cc. of milk into a small flask and add 25 cc. of neutral lead acetate solution. Connect the flask to a condenser, and heat the mixture with shaking, over a small flame until it begins to boil. Cool quickly under the tap. Filter the precipitate onto a small filter, collecting the filtrate in a 100 cc. volumetric flask. Repeat the filtration with the same filter until the filtrate is perfectly clear. Wash the precipitate on the filter with distilled water until the filtrate is made up to a volume of 100 cc. Determine the sugar in the solution so obtained by means of the polariscope. The 200-millimeter tube should be used, as the rotation will then give the sugar content of the 50 cc. of milk taken.

Determination of Fat

108.—*Babcock Method.*—See Exp. 22, page 33.

BLOOD[1]

109.—Centrifugalize blood and note the separation into three layers? What are these?

Blood Corpuscles

110.—(a) Examine a drop of blood under the microscope. Sketch the red and white corpuscles.

(b) Note the difference between the corpuscles of mammals, and those of birds and reptiles.

(c) Note the effect upon the red corpuscles produced by the addition of (1) water; (2) a concentrated solution of salt.

(d) Isotonic Solutions.—Starting with a 2.5% salt solution, determine by successive dilutions with an equal volume of water, the minimum percentage strength of a salt solution from which the corpuscles do not take up water.

Haemoglobin Crystals

111.—Place a drop of defibrinated rat's blood on a slide, add a drop or two of water, mix, and cover with a cover-glass. Sketch the crystals which separate after a few minutes. Or, add a few drops of ether to some blood in a test tube, shake thoroughly until the blood becomes "laky," and then place the tube on ice till crystals appear.

Absorption Spectra

112.—The principle of the spectroscope will be explained, and the absorption bands given by solutions of haemoglobin and its derivatives demonstrated. Prepare solutions for examination as follows:

1. Oxy-haemoglobin.—Use dilute blood (1 cc. defibrinated blood to 50 cc. of water).

2. Haemoglobin (reduced haemoglobin).—Add to blood a few drops of strong ammonium sulphide, or 1 or 2 drops of freshly prepared Stokes' reagent.[2]

Shake the solution of haemoglobin with air, and note the rapid change to oxy-haemoglobin. Change the same solution

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[1] Mathew's Chapter XII.

[2] Stokes's reagent is prepared as follows:—Dissolve 2 parts of ferrous sulphate, and 3 parts of tartaric acid in water, and add ammonia to distinct alkaline reaction. There should be no permanent precipitate.

of oxy-haemoglobin to haemoglobin and the reverse two or three times and note the facility with which haemoglobin takes up and loses oxygen.

3. Carbon-monoxide haemoglobin. — Pass a current of illuminating gas through a dilute oxy-haemoglobin solution for a few minutes and filter. Note the change of color. Try the effect on the solution of (1) ammonium sulphide; (2) Stokes' reagent; (3) potassium ferricyanide; (4) shaking with air. Note the stability of the compound.

4. Methaemoglobin. — Add to dilute defibrinated blood (1:15) two drops of a freshly prepared concentrated solution of potassium ferricyanide. Note the change. Note the effect produced by the addition of reducing agents.

5. Acid haematin.—Add a few drops of glacial acetic acid to dilute defibrinated blood and warm gently. The color becomes brownish owing to the formation of acid haematin.

6. Alkali haematin.—Add two or three drops of a strong NaOH solution to diluted blood, and warm gently till the color changes to a brownish green.

7. Reduced haematin, or haemochromogen.—Add to a solution of alkali-haematin a few drops of ammonium sulphide and warm gently; or, add to a solution of reduced haemoglobin a few drops of concentrated NaOH solution.

8. Haematoporphyrin (iron-free haematin).—To 10 cc. of concentrated sulphuric acid in a test tube add 5 drops of blood, and shake thoroughly. Note the wine-red or violet-red color and the two absorption bands. Dilute with a large excess of water, neutralize the acid with an alkali, dissolve the precipitated haematoporphyrin in ammonia, and note the four absorption bands.

Make drawings of all the bands seen and compare with a table of spectra.[1]

Haemin Crystals (Teichmann's Test)

113.—Place a bit of powdered dried blood on a glass side,

[1] Hawk's Practical Physiological Chemistry.

add a minute crystal of NaCl (fresh blood contains sufficient NaCl) and two drops of *glacial* acetic acid. Cover with a cover-glass and warm *gently* over a flame until bubbles appear. On cooling, dark-brown rhombic crystals, often crossed, separate (chloride of haematin). Similar crystals can be obtained by using an alkaline iodide or bromide in place of NaCl.

To apply this test for the detection of blood pigment in urine, coagulate the albumin present by heat, and filter. The blood pigment adheres to the precipitate and may be detected as above described, using the washed and dried precipitate. The haematin may also be precipitated by means of a solution of sodium tungstate acidified with acetic acid.

Coagulation of Blood

114.—(a) Observe the phenomena of coagulation as it takes place (a) in a test tube; (b) in a drop of blood, examined under the microscope.

(b) Add a few cc. of blood to 5 cc. of a 0.75% salt solution (normal saline) and filter or place in a test tube surrounded by ice till the corpuscles settle. Transfer the plasma to a watch-glass, and note the result. Conclusions?

Conditions Influencing Coagulation

115.—(a) Cold.—Receive blood into a test tube surrounded by ice. Note the effect on coagulation.

(b) Neutral salts.—Mix one volume of a 27% solution of $MgSO_4$ with four volumes of blood (salted plasma). Note the result. Dilute some of this salted plasma with 10 volumes of water. What takes place?

(c) Oxalate solutions.—Allow 100 cc. of blood to run into 5 cc. of a 6% solution of potassium oxalate. Mix. The blood remains fluid (oxalate plasma). Dilute some of this with water and note the result.

(d) Relation of calcium salts.—To oxalate plasma add a few drops of a 2% calcium chloride solution. What is the result? Explain.

Proteins of Blood-plasma

116.—

(a) Serum-albumin.

(b) Serium-globulin.

Using blood serum, separate and identify these two proteins.

(c) Fibrinogen.—Fibrinogen is a globulin found in blood, lymph, etc., along with paraglobulin. Like paraglobulin it responds to all the general precipitants and tests, and in addition gives the reactions with CO_2, dialysis, and $MgSO_4$. It is easily distinguished from paraglobulin by two reactions, viz., its power to coagulate, *i. e.*, to form fibrin when acted on by fibrin ferment, and its temperature of heat coagulation. Demonstrate these two properties in this way:

1. Take two specimens, one of salted plasma and one of serum. Dilute each five or ten times with water. The salted plasma will colt after a certain time, owing to the presence of the fibrinogen; the serum will not clot.

2. Take two other specimens, one of salted plasma and one of serum, and making use of the apparatus for the determination of the temperature of coagulation, heat them both slowly to 50°-60° C. The salted plasma will show a scanty precipitate of coagulated fibrinogen. The serum will not be affected. Dilute the heated specimen of salted plasma five to ten times; no clotting will occur, as all the fibrinogen has been removed.

Fibrin

117.—

(a) Note its physical properties.

(b) Note action of 0.2% hydrochloric acid.

(c) Apply the protein color tests.

118.—What are the proteins of plasma; serum; clot? What is the difference between serum and defibrinated blood?

BILE[1]

Color

119.—What is the difference between the color of human bile and ox bile? Explain.

Reaction

120.—Dilute some bile with four parts of water. Immerse a strip of red litmus paper, then remove and wash with water. Note the reaction.

Nucleo-albumin

121.—Dilute bile with twice its volume of water, filter if necessary, and add acetic acid. What is the precipitate? How distinguished from mucin?

122.—Filter (above) and test the filtrate for proteins. Report the result.

Separation of Bile Salts

123.—Mix 20 cc. of bile with animal charcoal to form a thick paste, and evaporate on the water-bath to complete dryness. Pulverize the residue in a mortar, transfer to a flask, add 25 cc of absolute alcohol, and heat on the water-bath for half an hour Filter. To the filtrate add ether till a permanent precipitate forms. Let the mixture stand for a day or two and then filter off the crystalline deposit of bile salts. Save the filtrate which contains cholesterin.

Pettenkofer's Test for Bile Acids

124.—Put some bile diluted with an equal volume of water in a wine glass, add a few drops of a 10% solution of cane sugar, then incline the glass and pour concentrated H_2SO_4 *carefully* down the side of the glass, so as to form a layer beneath the bile. Mix the two liquids *gradually* by stirring with a glass rod. A cherry red, changing to a reddish purple color, soon develops. The success of this test depends upon keeping the temperature below 70° C. Explain the reaction.

125.—Repeat above, using a dilute solution of bile salts.

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[2] Mathews. Page 406.

Bile Pigments

126.—(a) Gmelin's Test.—Take some bile in a wine glass and add **yellow** HNO_3 in the manner described for H_2SO_4 in No. 124. Notice the play of colors—beginning with green and passing through blue, violet, and red, to yellow—at the junction of the two liquids. Explain.

(b) Iodine Test.—Place 10 cc. of dilute bile in a test tube, and add slowly two or three cc. of dilute tincture of iodine, so that it forms an upper layer. A bright green ring forms at the line of contact. (Note: There are several modifications of Pettenkofer's test and of Gmelin's test.)

Cholesterin

127.—Examine under the microscope the crystals obtained by the cautious evaporation of the alcohol-ether filtrate of No. 123 or that obtained under No. 128.

For color reactions, refer to demonstrations.

Analysis of Biliary Calculi

128.—Pulverize, and extract several times with water to remove bile. Dry and extract with a mixture containing equal parts of alcohol and ether, which dissolves cholesterin. If there is a residue insoluble in the alcohol and ether mixture, treat it with dilute HCl. An effervescence indicates a carbonate ($CaCO_3$). Copper, if present, will be found in the HCl solution. Any insoluble residue still remaining is washed with water and treated with chloroform which dissolves bilirubin. For purification remove chloroform by evaporation, treat with alcohol and ether, re-dissolve in chloroform, filter if necessary, and evaporate till bilirubin begins to separate. Upon the addition of alcohol, bilirubin separates as an orange precipitate.

Action of Bile in Digestion

129.—(a) Take three test tubes. In one mix 10 cc. of bile and 2 cc. of neutral olive oil: in the second, 10 cc. of bile and 2 cc. of rancid olive oil; in the third, 10 cc. of water and 2 cc. of neutral oil. Shake and place in a water bath at 40° C. for some time. Note the extent and the permanency of the emulsion in each case.

(b) Into each of two funnels fit a filter-paper. Moisten one with water, and the other with bile, and into each pour an equal volume of olive oil. Set aside for twelve hours (with a beaker under each funnel). Do you notice any difference in the rate of filtration?

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